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<b>(54) Title:</b> SUBTILISIN BPN' VARIANTS HAVING DECREASED ADSORPTION AND INCREASED HYDROLYSIS		
<b>(57) Abstract</b>		
<p>The present invention relates to subtilisin BPN' variants having a modified amino acid sequence of wild-type BPN' amino acid sequence, the wild-type amino acid sequence comprising a first loop region, a second loop region, a third loop region, a fourth loop region and a fifth loop region; wherein the modified amino acid sequence comprises different amino acids than that occurring in wild-type subtilisin BPN' (i.e., substitution) at specifically identified positions in one or more of the loop regions whereby the BPN' variant has decreased adsorption to, and increased hydrolysis of, an insoluble substrate as compared to the wild-type subtilisin BPN'. The present invention also relates to the genes encoding such subtilisin BPN' variants. The present invention also relates to compositions comprising such subtilisin BPN' variants for cleaning a variety of surfaces.</p>		

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Subtilisin BPN' variants having decreased adsorption and increased hydrolysis

5

### TECHNICAL FIELD

10 The present invention relates to novel enzyme variants useful in a variety of cleaning compositions, and the genes encoding such enzyme variants.

### BACKGROUND

Enzymes make up the largest class of naturally occurring proteins. Each class of enzyme generally catalyzes (accelerates a reaction without being  
15 consumed) a different kind of chemical reaction. One class of enzymes known as proteases, are known for their ability to hydrolyze (break down a compound into two or more simpler compounds with the uptake of the H and OH parts of a water molecule on either side of the chemical bond cleaved) other proteins. This ability to hydrolyze proteins has been taken advantage of by incorporating  
20 naturally occurring and protein engineered proteases as an additive to laundry detergent preparations. Many stains on clothes are proteinaceous and wide-specificity proteases can substantially improve removal of such stains.

Unfortunately, the efficacy level of these proteins in their natural, bacterial environment, frequently does not translate into the relatively unnatural wash  
25 environment. Specifically, protease characteristics such as thermal stability, pH stability, oxidative stability and substrate specificity are not necessarily optimized for utilization outside the natural environment of the enzyme.

The amino acid sequence of the protease determines the characteristics of the protease. A change of the amino acid sequence of the protease may alter  
30 the properties of the enzyme to varying degrees, or may even inactivate the enzyme, depending upon the location, nature and/or magnitude of the change in the amino acid sequence. Several approaches have been taken to alter the wild-type amino acid sequence of proteases in an attempt to improve their properties, with the goal of increasing the efficacy of the protease in the wash  
35 environment. These approaches include altering the amino acid sequence to

enhance thermal stability and to improve oxidation stability under quite diverse conditions.

Despite the variety of approaches described in the art, there is a continuing need for new effective variants of proteases useful for cleaning a  
5 variety of surfaces.

#### Objects of the Present Invention

It is an object of the present invention to provide subtilisin enzyme variants having improved hydrolysis versus the wild-type of the enzyme.

It is also an object of the present invention to provide cleaning  
10 compositions comprising these subtilisin enzyme variants.

#### SUMMARY

The present invention relates to subtilisin BPN' variants having a modified amino acid sequence of wild-type BPN' amino acid sequence, the wild-type amino acid sequence comprising a first loop region, a second loop region, a  
15 third loop region, a fourth loop region and a fifth loop region; wherein the modified amino acid sequence comprises different amino acids than that occurring in wild-type subtilisin BPN' (i.e., substitution) at specifically identified positions in one or more of the loop regions whereby the BPN' variant has decreased adsorption to, and increased hydrolysis of, an insoluble substrate as  
20 compared to the wild-type subtilisin BPN'. The present invention also relates to the genes encoding such subtilisin BPN' variants. The present invention also relates to compositions comprising such subtilisin BPN' variants for cleaning a variety of surfaces.

#### DESCRIPTION

##### I. Subtilisin Variants

This invention pertains to subtilisin enzymes, in particular BPN', that have been modified by mutating the various nucleotide sequences that code for the enzyme, thereby modifying the amino acid sequence of the enzyme. The modified subtilisin enzymes (hereinafter, "BPN' variants") of the present  
30 invention have decreased adsorption to and increased hydrolysis of an insoluble substrate as compared to the wild-type subtilisin. The present invention also pertains to the mutant genes encoding for such BPN' variants.

The subtilisin enzymes of this invention belong to a class of enzymes known as proteases. A protease is a catalyst for the cleavage of peptide bonds.  
35 One type of protease is a serine protease. A serine protease is distinguished by



the fact that there is an essential serine residue at the active site.

The observation that an enzyme's rate of hydrolysis of soluble substrates increases with enzyme concentration is well documented. It would therefore seem plausible that for surface bound substrates, such as is encountered in many cleaning applications, the rate of hydrolysis would increase with increasing surface concentration. This has been shown to be the case. (Brode, P.F. III and D. S. Rauch, *LANGMUIR*, "Subtilisin BPN': Activity on an Immobilized Substrate", Vol. 8, pp. 1325-1329 (1992)). In fact, a linear dependence of rate upon surface concentration was found for insoluble substrates when the surface concentration of the enzyme was varied. (Rubingh, D. N. and M. D. Bauer, "Catalysis of Hydrolysis by Proteases at the Protein-Solution Interface," in *POLYMER SOLUTIONS, BLENDS AND INTERFACES*, Ed. by I. Noda and D. N. Rubingh, Elsevier, p. 464 (1992)). Surprisingly, when seeking to apply this principle in the search for variant proteases which give better cleaning performance, we did not find that enzymes which adsorb more give better performance. In fact, we surprisingly determined the opposite to be the case: decreased adsorption by an enzyme to a substrate resulted in increased hydrolysis of the substrate (i.e., better cleaning performance).

While not wishing to be bound by theory, it is believed that improved performance, when comparing one variant to another, is a result of the fact that enzymes which adsorb less are also less tightly bound and therefore more highly mobile on the surface from which the insoluble protein substrate is to be removed. At comparable enzyme solution concentrations, this increased mobility is sufficient to outweigh any advantage that is conferred by delivering a higher concentration of enzyme to the surface.

The mutations described herein are designed to change (i.e., decrease) the adsorption of the enzyme to surface-bound soils. In BPN', certain amino acids form exterior loops on the enzyme molecule. For purposes of discussion, these loops shall be referred to as first, second, third, fourth and fifth loop regions. Specifically, positions 59-66 form the first loop region; positions 95-107 form the second loop region; positions 126-133 form the third loop region; positions 154-167 form the fourth loop region; positions 187-191 form the fifth loop region; and positions 199-220 form the sixth loop region (position numbering analogous to positions in the amino acid sequence for wild-type subtilisin BPN' (SEQ ID NO:1)).

It believed that these loop regions play a significant role in the adsorption of the enzyme molecule to a surface-bound peptide, and specific mutations in one or more of these loop regions will have a significant effect on this adsorption. While not wishing to be bound by theory, it is believed that the loop regions are important to the adsorption of the BPN' molecule for at least two reasons. First, the amino acids which comprise the loop regions can make close contacts with any surfaces to which the molecule is exposed. Second, the proximity of the loop regions to the active-site and binding pocket of the BPN' molecule gives them a role in the catalytically productive adsorption of the enzyme to surface-bound substrates (peptides/protein soils).

As used herein, "variant" means an enzyme having an amino acid sequence which differs from that of wild-type.

As used herein, "mutant BPN' gene" means a gene coding for a BPN' variant.

As used herein, "wild-type subtilisin BPN'" refers to a subtilisin enzyme represented by SEQ ID NO:1. The amino acid sequence for subtilisin BPN' is further described by Wells, J. A., E. Ferrari, D. J. Henner, D. A. Estell and E. Y. Chen, NUCLEIC ACIDS RESEARCH, Vol. II, 7911-7925 (1983), incorporated herein by reference.

As used herein, the term "wild-type amino acid sequence" encompasses SEQ ID NO:1 as well as SEQ ID NO:1 having modifications to the amino acid sequence other than at any of positions 59-66, 95-107, 126-133, 154-167, 187-191 and 199-220.

As used herein, "more hydrophilic amino acid" refers to any other amino acid having greater hydrophilicity than a subject amino acid with reference to the hydrophilicity table below. The following hydrophilicity table (Table 1) lists amino acids in descending order of increasing hydrophilicity (see Hopp, T.P., and Woods, K.R., "Prediction of Protein Antigenic Determinants from Amino Acid Sequences", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE USA, Vol. 78, pp. 3824-3828, 1981, incorporated herein by reference).

TABLE 1

Amino Acid	Hydrophilicity Value
Trp	-3.4
Phe	-2.5
Tyr	-2.3
Leu, Ile	-1.8
Val	-1.5
Met	-1.3
Cys	-1.0
Ala, His	-0.5
Thr	-0.4
Pro, Gly	-0.0
Gln, Asn	0.2
Ser	0.3
Arg <sup>+</sup> , Lys <sup>+</sup> , Glu <sup>-</sup> , Asp <sup>-</sup>	3.0

Table 1 also indicates which amino acids carry a charge (this characteristic being based on a pH of from about 8-9). The positively charged amino acids are Arg and Lys, the negatively charged amino acids are Glu and Asp, and the remaining amino acids are neutral. In a preferred embodiment of the present invention, the substituting amino acid is either neutral or negatively charged, more preferably negatively charged (i.e., Glu or Asp).

Therefore, for example, the statement "substitute Gln with an equally or more hydrophilic amino acid which is neutral or has a negative charge" means Gln would be substituted with Asn (which is equally hydrophilic to Gln), or Ser, Glu or Asp (which are more hydrophilic than Gln); each of which are neutral or have a negative charge, and have a greater hydrophilicity value as compared to Gln. Likewise, the statement "substitute Pro with a more hydrophilic amino acid which is neutral or has a negative charge" means Pro would be substituted with Gln, Asn, Ser, Glu or Asp.

In one embodiment of the present invention, the BPN' variant has a modified amino acid sequence of wild-type amino acid sequence, wherein the modified amino acid sequence comprises a substitution at one or more positions in one or more of the first, second, third, fourth or fifth loop regions; whereby the BPN' variant has decreased adsorption to, and increased hydrolysis of, an insoluble substrate as compared to the wild-type subtilisin BPN'.

In another embodiment of the present invention, the BPN' variant further comprises one or more substitutions to the sixth loop region.

In a preferred embodiment of the present invention, the substituting amino acid for one or more of the positions in one or more of the loop regions is, with reference to Table 1, neutral or negatively charged and equally or more hydrophylic, preferably more hydrophylic, than the amino acid at the subject position in the wild-type amino acid sequence.

**A. Substitutions in the First Loop Region**

When a substitution occurs in the first loop region, the substitution occurs at one or more of positions 59, 60, 61, 62, 63, 65 or 66.

When a substitution occurs at position 59, the substituting amino acid is Asn, Asp, Glu or Ser.

When a substitution occurs at position 60, the substituting amino acid is Glu.

When a substitution occurs at position 61, the substituting amino acid is Asp, Gln, Glu or Ser.

When a substitution occurs at position 62, the substituting amino acid is Asp, Gln, Glu or Ser.

When a substitution occurs at position 63, the substituting amino acid is Asp or Glu.

When a substitution occurs at position 65, the substituting amino acid is Asn, Asp, Gln, Glu, Pro or Ser.

When a substitution occurs at position 66, the substituting amino acid is Asn, Asp, Gln, Glu, Gly, Pro or Ser.

**B. Substitutions in the Second Loop Region**

When a substitution occurs in the second loop region, the substitution occurs at one or more of positions 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106 or 107.

When a substitution occurs at position 95, the substituting amino acid is Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Met, Pro, Ser or Thr.

When a substitution occurs at position 96, the substituting amino acid is Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Met, Pro, Ser, Thr or Val.

When a substitution occurs at position 97, the substituting amino acid is Asn, Asp, Gln, Glu, Pro or Ser.

When a substitution occurs at position 98, the substituting amino acid is Asn, Asp, Gln, Glu, Gly, His, Pro, Ser or Thr.

When a substitution occurs at position 99, the substituting amino acid is

Glu.

When a substitution occurs at position 100, the substituting amino acid is Asn, Asp, Gln, Glu, Pro or Ser.

When a substitution occurs at position 101, the substituting amino acid is  
5 Asp or Glu.

When a substitution occurs at position 102, the substituting amino acid is Asn, Asp, Gln, Glu, Pro or Ser.

When a substitution occurs at position 103, the substituting amino acid is Asn, Asp, Glu or Ser.

10 When a substitution occurs at position 104, the substituting amino acid is Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Met, Pro, Ser, Thr or Val.

When a substitution occurs at position 105, the substituting amino acid is Asp or Glu.

When a substitution occurs at position 106, the substituting amino acid is  
15 Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Pro, Ser, Thr, Tyr or Val.

When a substitution occurs at position 107, the substituting amino acid is Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Leu, Met, Pro, Ser, Thr or Val.

C. Substitutions in the Third Loop Region

20 When a substitution occurs in the third loop region, the substitution occurs at one or more of positions 126, 127, 128, 129, 130, 131, 132 or 133.

When a substitution occurs at position 126, the substituting amino acid is Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Met, Pro, Ser, Thr or Val.

When a substitution occurs at position 127, the substituting amino acid is  
25 Asn, Asp, Gln, Glu, Pro or Ser.

When a substitution occurs at position 128, the substituting amino acid is Asn, Asp, Gln, Glu, Gly or Ser.

When a substitution occurs at position 129, the substituting amino acid is Asn, Asp, Gln, Glu, Gly or Ser.

30 When a substitution occurs at position 130, the substituting amino acid is Asp or Glu.

When a substitution occurs at position 131, the substituting amino acid is Asn, Asp, Gln, Glu, Gly or Ser.

When a substitution occurs at position 132, the substituting amino acid is  
35 Asp or Glu.

When a substitution occurs at position 133, the substituting amino acid is Asn, Asp, Gln, Glu, Gly, His, Pro, Ser or Thr.

**D. Substitutions in the Fourth Loop Region**

When a substitution occurs in the fourth loop region, the substitution occurs at one or more of positions 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166 or 167.

When a substitution occurs at position 154, the substituting amino acid is Asn, Asp, Gln, Glu, Pro or Ser.

When a substitution occurs at position 155, the substituting amino acid is Asp, Gln, Glu or Ser.

When a substitution occurs at position 156, the substituting amino acid is Asp.

When a substitution occurs at position 157, the substituting amino acid is Asn, Asp, Gln, Glu, Pro or Ser.

When a substitution occurs at position 158, the substituting amino acid is Asn, Asp, Gln, Glu, Gly, Pro or Ser.

When a substitution occurs at position 159, the substituting amino acid is Asp or Glu.

When a substitution occurs at position 160, the substituting amino acid is Asn, Asp, Gln, Glu, Pro or Ser.

When a substitution occurs at position 161, the substituting amino acid is Asp or Glu.

When a substitution occurs at position 162, the substituting amino acid is Asp or Glu.

When a substitution occurs at position 163, the substituting amino acid is Asp or Glu.

When a substitution occurs at position 164, the substituting amino acid is Asn, Asp, Gln, Glu, Gly, Pro or Ser.

When a substitution occurs at position 165, the substituting amino acid is Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Met, Pro, Ser or Thr.

When a substitution occurs at position 166, the substituting amino acid is Asn, Asp, Gln, Glu, Pro or Ser.

When a substitution occurs at position 167, the substituting amino acid is Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Met, Pro, Ser, Thr or Val.

**E. Substitutions in the Fifth Loop Region**

When a substitution occurs in the fifth loop region, the substitution occurs at one or more of positions 187, 188, 189, 190 or 191.

When a substitution occurs at position 187, the substituting amino acid is  
5 Asn, Asp, Gln, Glu, Gly, His, Pro, Ser and Thr.

When a substitution occurs at position 188, the substituting amino acid is Asp or Glu.

When a substitution occurs at position 189, the substituting amino acid is Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Met, Pro, Ser, Thr, Tyr or Val.

10 When a substitution occurs at position 190, the substituting amino acid is Asp or Glu.

When a substitution occurs at position 191, the substituting amino acid is Asp or Glu.

**F. Substitutions in the Sixth Loop Region**

15 When a substitution occurs in the sixth loop region, the substitution occurs at one or more of positions 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219 or 220.

When a substitution occurs at position 199, the substituting amino acid for position 199 is Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

20 When a substitution occurs at position 200, the substituting amino acid for position 200 is His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

When a substitution occurs at position 201, the substituting amino acid for position 201 is Gly, Gln, Asn, Ser, Asp or Glu.

25 When a substitution occurs at position 202, the substituting amino acid for position 202 is Pro, Gln, Asn, Ser, Asp or Glu.

When a substitution occurs at position 203, the substituting amino acid for position 203 is Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

When a substitution occurs at position 204, the substituting amino acid for position 204 is Asp, or Glu.

30 When a substitution occurs at position 205, the substituting amino acid for position 205 is Leu, Val, Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

When a substitution occurs at position 206, the substituting amino acid for position 206 is Pro, Asn, Ser, Asp, or Glu.

35 When a substitution occurs at position 207, the substituting amino acid for

position 207 is Asp or Glu.

When a substitution occurs at position 208, the substituting amino acid for position 208 is Pro, Gly, Gln, Asn, Ser, Asp or Glu.

When a substitution occurs at position 209, the substituting amino acid for position 209 is Ile, Val, Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

When a substitution occurs at position 210, the substituting amino acid for position 210 is Ala, Gly, Gln, Asn, Ser, Asp or Glu.

When a substitution occurs at position 211, the substituting amino acid for position 211 is Ala, Pro, Gln, Asn, Ser, Asp or Glu.

When a substitution occurs at position 212, the substituting amino acid for position 212 is Gln, Ser, Asp or Glu.

When a substitution occurs at position 213, the substituting amino acid for position 213 is Trp, Phe, Tyr, Leu, Ile, Val, Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

When a substitution occurs at position 214, the substituting amino acid for position 214 is Phe, Leu, Ile, Val, Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

When a substitution occurs at position 215, the substituting amino acid for position 215 is Thr, Pro, Gln, Asn, Ser, Asp or Glu.

When a substitution occurs at position 216, the substituting amino acid for position 216 is His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

When a substitution occurs at position 217, the substituting amino acid for position 217 is Leu, Ile, Val, Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

When a substitution occurs at position 218, the substituting amino acid for position 218 is Gln, Ser, Asp or Glu.

When a substitution occurs at position 219, the substituting amino acid for position 219 is Pro, Gln, Asn, Ser, Asp or Glu.

When a substitution occurs at position 220, the substituting amino acid for position 220 is Pro, Gly, Gln, Asn, Ser, Asp or Glu.

#### G. Preparation of enzyme variants

##### Example 1

##### Mutant BPN' Genes

A phagemid (pSS-5) containing the wild type subtilisin BPN' gene



(Mitchinson, C. and J. A. Wells, (1989), "Protein Engineering of Disulfide Bonds in Subtilisin BPN", *BIOCHEMISTRY*, Vol. 28, pp. 4807-4815) is transformed into *Escherichia coli ung*-strain CJ236 and a single stranded uracil-containing DNA template is produced using the VCSM13 helper phage (Kunkel, T.A., J.D. Roberts and R.A. Zakour, "Rapid and efficient site-specific mutagenesis without phenotypic selection", *METHODS IN ENZYMOLOGY*, Vol. 154, pp. 367-382, (1987); as modified by Yuckenberg, P.D., F. Witney, J. Geisselsoder and J. McClary, "Site-directed in vitro mutagenesis using uracil-containing DNA and phagemid vectors", *DIRECTED MUTAGENESIS - A PRACTICAL APPROACH*, ed. M.J. McPherson, pp. 27-48, (1991); both of which are incorporated herein by reference). A single primer site-directed mutagenesis modification of the method of Zoller and Smith (Zoller, M.J., and M. Smith, "Oligonucleotide-directed mutagenesis using M13-derived vectors: an efficient and general procedure for the production of point mutations in any fragment of DNA", *NUCLEIC ACIDS RESEARCH*, Vol. 10, pp. 6487-6500, (1982), incorporated herein by reference) is used to produce all mutants (basically as presented by Yuckenberg, *et al.*, 1991, above). Oligonucleotides are made using an Applied Biosystem Inc. 380B DNA synthesizer. Mutagenesis reaction products are transformed into *Escherichia coli* strain MM294 (American Type Culture Collection *E. Coli*. 33625). All mutants are confirmed by DNA sequencing and the isolated DNA is transformed into the *Bacillus subtilis* expression strain BG2036 (Yang, M. Y., E. Ferrari and D. J. Henner, (1984), "Cloning of the Neutral Protease Gene of *Bacillus subtilis* and the Use of the Cloned Gene to Create an *In Vitro*-derived Deletion Mutation", *JOURNAL OF BACTERIOLOGY*, Vol. 160, pp. 15-21). For some of the mutants a modified pSS-5 with a frameshift-stop codon mutation at amino acid 217 is used to produce the uracil template. Oligonucleotides are designed to restore the proper reading frame at position 217 and also encoded for random substitutions at positions 59, 60, 61, 62, 63, 64, 65, 66; 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107; 126, 127, 128, 129, 130, 131, 132, 133; 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167; 187, 188, 189, 190, 191; 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219 and 220 (equimolar and/or variable mixtures of all four nucleotides for all three bases at these codons). Mutations that correct for the frameshift-stop and produce a functional enzyme are identified by their ability to digest casein. The random substitutions are determined by DNA sequencing.

## Example 2

### Fermentation

The *Bacillus subtilis* cells (BE2036) containing a subtilisin mutant of interest are grown to mid-log phase in a one liter culture of LB-glucose broth and inoculated into a Biostat ED fermenter (B. Braun Biotech, Inc., Allentown, Pennsylvania) in a total volume of 10 liters. The fermentation media contains Yeast Extract, starch, antifoam, buffers and trace minerals (see FERMENTATION: A PRACTICAL APPROACH, Ed. B. McNeil and L. M. Harvey, 1990). The broth is kept at a constant pH of 7.0 during the fermentation run. Chloramphenicol is added for antibiotic selection of mutagenized plasmid. The cells are grown overnight at 37°C to an A<sub>600</sub> of about 60 and harvested.

## Example 3

### Purification

The fermentation broth is taken through the following steps to obtain pure enzyme. The broth is cleared of *Bacillus subtilis* cells by centrifugation, and clarified by removing fine particulates with a 100K cutoff membrane. This is followed by concentration on a 10K cutoff membrane, and flow dialysis to reduce the ionic strength and adjust the pH to 5.5 using 0.025M MES buffer (2-(*N*-morpholino)ethanesulfonic acid). The enzyme is further purified by loading it onto either a cation exchange chromatography column or an affinity adsorption chromatography column and eluting it from the column with a NaCl or a propylene glycol gradient (see Scopes, R. K., PROTEIN PURIFICATION PRINCIPLES AND PRACTICE, Springer-Verlag, New York (1984), incorporated herein by reference).

The *p*NA assay (DelMar, E.G., C. Largman, J.W. Brodrick and M.C. Geokas, ANAL. BIOCHEM., Vol. 99, pp. 316-320, (1979), incorporated herein by reference) is used to determine the active enzyme concentration for fractions collected during gradient elution. This assay measures the rate at which *p*-nitroaniline is released as the enzyme hydrolyzes the soluble synthetic substrate, succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide (sAAPF-*p*NA). The rate of production of yellow color from the hydrolysis reaction is measured at 410 nm on a spectrophotometer and is proportional to the active enzyme concentration. In addition, absorbance measurements at 280 nm are used to determine the total protein concentration. The active enzyme/total-protein ratio gives the enzyme purity, and is used to identify fractions to be

pooled for the stock solution.

To avoid autolysis of the enzyme during storage, an equal weight of propylene glycol is added to the pooled fractions obtained from the chromatography column. Upon completion of the purification procedure the  
5 purity of the stock enzyme solution is checked with SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and the absolute enzyme concentration is determined via an active site titration method using trypsin inhibitor type II-T: turkey egg white purchased from Sigma Chemical Company (St. Louis, Missouri). The measured conversion factors will show which changes  
10 made in the enzyme molecule at the various positions result in the enzyme variant having increased activity over the wild-type, against the soluble substrate pNA.

In preparation for use, the enzyme stock solution is eluted through a Sephadex-G25 (Pharmacia, Piscataway, New Jersey) size exclusion column to  
15 remove the propylene glycol and exchange the buffer. The MES buffer in the enzyme stock solution is exchanged for 0.1 M Tris buffer (Tris(hydroxymethyl-aminomethane) containing 0.01M  $\text{CaCl}_2$  and pH adjusted to 8.6 with HCl. All experiments are carried out at pH 8.6 in Tris buffer thermostated at 25°C.

#### H. Characterization of enzyme variants

20

##### Example 4

##### Model Surface Preparation

Aminopropyl controlled pore glass (CPG) purchased from CPG Inc. (Fairfield, New Jersey) is used as a support for covalently attaching the sAAPF-pNA substrate purchased from Bachem, Inc. (Torrence, California). The reaction  
25 is carried out in dimethyl sulfoxide and (1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide hydrochloride) (EDC) is used as a coupling agent. Upon completion (monitored by pNA assay), the excess solvent is removed, and the CPG:sAAPF-pNA is rinsed with dimethyl sulfoxide (DMSO) and doubly-distilled water. This is followed by oven drying with a  $\text{N}_2$  purge at about 70°C. The  
30 reaction scheme and preparation of the immobilized substrate are conducted as described by Brode, P.F. III, and D.S. Rauch, "Subtilisin BPN": Activity on an Immobilized Substrate," LANGMUIR, Vol. 8, p. 1325-1329, (1992), incorporated herein by reference.

The CPG surface will have  $62,000 \pm 7,000$  pNA molecules/ $\mu\text{m}^2$ . The  
35 surface area will remain unchanged from the value of  $50.0\text{m}^2/\text{g}$  reported by CPG

Inc. for the CPG as received. This suggests that the procedure used to add sAAPF-pNA to CPG does not damage the porous structure (mean diameter is 486 Å).

#### Example 5

5

##### Surface Hydrolysis Assay

Using CPG:sAAPF-pNA, adsorption of an enzyme variant and hydrolysis of a CPG-bound peptide can be measured in a single experiment. A small volume of enzyme variant stock solution is added to a flask containing Tris buffer and CPG:sAAPF-pNA which has been degassed. The flask is shaken on a wrist-action shaker for a period of 90 minutes during which the shaker is stopped at various time intervals (for example, every 2 minutes during the early stages of adsorption hydrolysis - e.g., the first 20 minutes - and every 10 minutes towards the end of the experiment). The CPG:sAAPF-pNA is allowed to settle and the solution is sampled. Both the experimental procedure and the calculation of the adsorption and hydrolysis are conducted as described by Brode *et al.*, 1992, above.

All enzymes are monitored for stability against autolysis and should show no appreciable autolytic loss over the time course of this experiment. Therefore, enzyme adsorption can be determined by measuring solution depletion. The difference between the initial enzyme variant concentration and the concentration measured at each individual time point gives the amount of enzyme variant adsorbed. The amount of pNA hydrolyzed from the surface is measured by taking an absorbance reading on an aliquot of the sample at 410 nm. The total amount of pNA hydrolyzed is calculated by adding the amount sampled and the amount remaining in the flask. This value is corrected by subtracting the amount of pNA that is hydrolyzed by Tris buffer at pH 8.6 when no enzyme is present. This base-hydrolysis ranges from 7-29% of the total hydrolysis depending on the efficiency of the enzyme.

#### Example 6

30

##### Soluble Substrate Kinetic Analysis

The rates of hydrolysis of the soluble substrate sAAPF-pNA are monitored by measuring the adsorbance increase as a function of time at 410 nm on a DU-70 spectrophotometer. The enzyme concentration is held constant and is prepared to be in the range of 6-10 nanomolar while the substrate concentration is varied from 90-700 µM sAAPF-pNA for each kinetic determination. An

adsorbance data point is taken each second over a period of 900 seconds and the data are transferred to a Lotus™ spreadsheet (Lotus Development Corporation, Cambridge, Massachusetts). Analysis for kinetic parameters is conducted by the standard Lineweaver Burk analysis in which the data in the  
 5 initial part of the run (generally the first minute) are fit to a linear regression curve to give  $v_0$ . The  $v_0$  and  $s_0$  data are plotted in the standard inverse fashion to give  $K_M$  and  $k_{cat}$ .

I. Example BPN' variants

BPN' variants of the present invention which have decreased adsorption  
 10 to and increased hydrolysis of surface bound substrates are exemplified in Tables 2-25, below. In describing the specific mutations, the original amino acid occurring in wild-type is given first, the position number second, and the substituted amino acid third.

TABLE 2

15	Loop 1 - Single Mutation Variants
	Gln59Asn
	Gln59Asp
	Gln59Glu
	Gln59Ser
20	Asp60Glu
	Asn61Asp
	Asn61Gln
	Asn61Glu
	Asn61Ser
25	Asn62Asp
	Asn62Gln
	Asn62Glu
	Asn62Ser
	Ser63Asp
30	Ser63Glu
	Gly65Asn
	Gly65Asp
	Gly65Gln
	Gly65Glu
35	Gly65Pro
	Gly65Ser
	Thr66Asn
	Thr66Asp
	Thr66Gln
40	Thr66Glu
	Thr66Gly
	Thr66Pro
	Thr66Ser

TABLE 3

Loop 1 - Double Mutation Variants	
5	Gln59Ser + Asn62Glu
	Asp60Glu + Asn61Ser
	Asn61Glu + Asn62Ser
10	Gln59Ser + Gly65Gln
	Asn61Gln + Gly65Asn
	Asn61Ser + Asn62Asp
	Gln59Glu + Asn61Gln
	Asp60Glu + Gly65Gln
15	Gln59Asp + Gly65Pro
	Asn61Asp + Gly65Asn
	Gln59Ser + Asn62Asp
	Gln59Asn + Gly65Gln
	Asn62Asp + Thr66Gly
20	Gln59Asn + Asn62Glu
	Asn61Ser + Ser63Glu
	Gln59Ser + Asp60Glu
	Asp60Glu + Thr66Gln
	Asn61Glu + Thr66Gly
25	Asp60Glu + Asn62Gln
	Asn62Gln + Gly65Pro
	Asn61Ser + Thr66Ser
	Asp60Glu + Gly65Pro
	Ser63Glu + Gly65Pro
30	Asp60Glu + Thr66Ser
	Gln59Ser + Asn61Glu
	Asn62Asp + Gly65Gln
	Asn61Gln + Ser63Asp
	Gln59Asp + Gly65Asn
35	Ser63Asp + Thr66Pro
	Ser63Glu + Thr66Asn
	Asn62Glu + Thr66Asn
	Asn61Asp + Gly65Ser
	Gly65Pro + Thr66Ser
40	Gln59Ser + Asn62Ser
	Asp60Glu + Gly65Ser
	Ser63Asp + Gly65Ser
	Asn61Gln + Ser63Glu
	Asn61Asp + Asn62Ser
45	Gln59Glu + Gly65Pro
	Gln59Ser + Asn61Asp
	Gln59Asp + Asn62Ser
	Gln59Asn + Gly65Ser
	Ser63Glu + Thr66Ser
	Asn61Ser + Ser63Asp
	Asn62Ser + Gly65Pro

TABLE 4

Loop 1 - Triple Mutation Variants			
5	Gln59Ser + Ser63Asp + Gly65Pro		
	Asn62Gln + Gly65Ser + Thr66Asp		
	Gln59Ser + Asp60Glu + Thr66Gln		
	Gln59Asn + Ser63Glu + Thr66Pro		
10	Asn61Ser + Gly65Asn + Thr66Glu		
	Ser63Glu + Gly65Ser + Thr66Asn		
	Asn62Asp + Gly65Ser + Thr66Gly		
	Gln59Ser + Asn62Asp + Thr66Pro		
15	Gln59Ser + Asp60Glu + Asn61Gln		
	Asn61Gln + Ser63Asp + Gly65Ser		
	Asn62Glu + Gly65Asn + Thr66Gln		
	Asp60Glu + Gly65Asn + Thr66Ser		
20	Asn62Ser + Ser63Asp + Thr66Gln		
	Gln59Asp + Asn62Gln + Gly65Pro		
	Asn62Ser + Ser63Glu + Thr66Gly		
	Asn61Asp + Asn62Ser + Gly65Asn		
25	Asp60Glu + Asn61Gln + Asn62Ser		
	Asp60Glu + Asn61Gln + Gly65Ser		
	Asp60Glu + Gly65Pro + Thr66Asn		
	Gln59Ser + Asn61Glu + Asn62Asp		
30	Asn61Asp + Asn62Asp + Gly65Pro		
	Asn61Glu + Asn62Glu + Thr66Gln		
	Gln59Asp + Asp60Glu + Thr66Gln		
	Gln59Asp + Asp60Glu + Thr66Pro		
35	Asn62Asp + Ser63Asp + Gly65Asn		
	Asn62Glu + Ser63Glu + Gly65Asn		
	Asn62Asp + Ser63Glu + Gly65Gln		
	Gln59Ser + Asn62Asp + Ser63Glu		
40	Asn62Glu + Ser63Asp + Gly65Ser		
	Asn61Asp + Asn62Asp + Ser63Glu		
	Gln59Glu + Asp60Glu + Asn61Glu		
	Asp60Glu + Asn62Glu + Ser63Asp		
45	Asp60Glu + Asn61Glu + Ser63Glu		
	Gln59Ser + Asp60Glu + Asn62Glu		

TABLE 5

Loop 1 - Quadruple Mutation Variants			
40	Gln59Ser + Asp60Glu + Gly65Gln + Thr66Gln		
	Gln59Ser + Asn62Ser + Ser63Asp + Gly65Gln		
	Asp60Glu + Asn62Ser + Gly65Pro + Thr66Gln		
	Asn62Gln + Ser63Glu + Gly65Pro + Thr66Gln		
45	Asn61Gln + Asn62Gln + Ser63Asp + Gly65Pro		
	Gln59Asn + Asp60Glu + Asn61Gln + Gly65Asn		
	Gln59Glu + Asn62Ser + Gly65Pro + Thr66Ser		

	Gln59Asn + Asn61Asp + Asn62Asp + Thr66Asn
	Gln59Asp + Asp60Glu + Asn62Ser + Gly65Ser
	Asn61Gln + Asn62Asp + Ser63Glu + Thr66Gln
	Asp60Glu + Asn61Asp + Asn62Glu + Gly65Ser
5	Asn61Asp + Asn62Glu + Ser63Glu + Thr66Ser
	Asn61Asp + Asn62Glu + Ser63Asp + Gly65Ser
	Gln59Glu + Asp60Glu + Asn61Asp + Gly65Ser
	Asp60Glu + Asn62Asp + Ser63Glu + Thr66Pro
	Asp60Glu + Asn62Glu + Ser63Glu + Thr66Asn
10	Asp60Glu + Asn62Glu + Ser63Asp + Gly65Ser
	Asp60Glu + Asn61Asp + Ser63Glu + Thr66Asn
	Gln59Ser + Asp60Glu + Asn61Asp + Ser63Asp
	Asp60Glu + Asn61Asp + Ser63Asp + Gly65Pro
	Asp60Glu + Asn61Asp + Ser63Asp + Thr66Gly
15	Asp60Glu + Asn61Asp + Ser63Glu + Gly65Asn
	Gln59Ser + Asp60Glu + Asn62Asp + Thr66Gly
	Asp60Glu + Asn62Asp + Gly65Ser + Thr66Pro
	Asp60Glu + Asn61Gln + Asn62Glu + Gly65Ser
	Gln59Ser + Asp60Glu + Asn62Asp + Gly65Gln
20	Asp60Glu + Asn61Ser + Asn62Gln + Ser63Glu
	Asp60Glu + Asn61Ser + Ser63Asp + Thr66Pro
	Gln59Ser + Asp60Glu + Asn61Gln + Ser63Glu
	Asp60Glu + Ser63Glu + Gly65Ser + Thr66Asn
	Gln59Asn + Asp60Glu + Ser63Asp + Gly65Gln
25	Asp60Glu + Ser63Glu + Gly65Pro + Thr66Ser

TABLE 6

## Loop 2 - Single Mutation Variants

	Val95Ala
30	Val95Asn
	Val95Asp
	Val95Cys
	Val95Gln
	Val95Glu
35	Val95Gly
	Val95His
	Val95Met
	Val95Pro
	Val95Ser
40	Val95Thr
	Leu96Ala
	Leu96Asn
	Leu96Asp
	Leu96Cys
45	Leu96Gln
	Leu96Glu
	Leu96Gly
	Leu96His
	Leu96Ile



	Leu96Met
	Leu96Pro
	Leu96Ser
	Leu96Thr
5	Leu96Val
	Gly97Asn
	Gly97Asp
	Gly97Gln
	Gly97Glu
10	Gly97Pro
	Gly97Ser
	Ala98Asn
	Ala98Asp
	Ala98Gln
15	Ala98Glu
	Ala98Gly
	Ala98His
	Ala98Pro
	Ala98Ser
20	Ala98Thr
	Asp99Glu
	Gly100Asn
	Gly100Asp
	Gly100Gln
25	Gly100Glu
	Gly100Pro
	Gly100Ser
	Ser101Asp
	Ser101Glu
30	Gly102Asn
	Gly102Asp
	Gly102Gln
	Gly102Glu
	Gly102Pro
35	Gly102Ser
	Gln103Asn
	Gln103Asp
	Gln103Glu
	Gln103Ser
40	Tyr104Ala
	Tyr104Asn
	Tyr104Asp
	Tyr104Cys
	Tyr104Gln
45	Tyr104Glu
	Tyr104Gly
	Tyr104His
	Tyr104Ile
	Tyr104Leu
50	Tyr104Met

	Tyr104Pro
	Tyr104Ser
	Tyr104Thr
	Tyr104Val
5	Ser105Asp
	Ser105Glu
	Trp106Ala
	Trp106Asn
	Trp106Asp
10	Trp106Cys
	Trp106Gln
	Trp106Glu
	Trp106Gly
	Trp106His
15	Trp106Ile
	Trp106Leu
	Trp106Met
	Trp106Phe
	Trp106Pro
20	Trp106Ser
	Trp106Thr
	Trp106Tyr
	Trp106Val
	Ile107Ala
25	Ile107Asn
	Ile107Asp
	Ile107Cys
	Ile107Gln
	Ile107Glu
30	Ile107Gly
	Ile107His
	Ile107Leu
	Ile107Met
	Ile107Pro
35	Ile107Ser
	Ile107Thr
	Ile107Val

TABLE 7

40	Loop 2 - Double Mutation Variants
	Val 95Gln + Ser101Glu
	Gly 97Ser + Gly100Gln
	Ser105Glu + Trp106Gly
	Asp 99Glu + Gln103Asn
45	Ala 98Gln + Trp106Thr
	Gly 97Asp + Ile107Thr
	Gly100Ser + Gly102Gln
	Leu 96Ser + Ser101Glu
	Asp 99Glu + Ile107Ala

	Leu 96Asn + Asp 99Glu
	Gly102Gln + Trp106Asp
	Tyr104Leu + Trp106Glu
	Tyr104Pro + Ile107Asp
5	Gly 97Ser + Ser101Asp
	Gly100Pro + Ser101Glu
	Val 95Asn + Ala 98Asp
	Val 95Met + Ile107Gly
	Asp 99Glu + Trp106Cys
10	Gly100Asn + Trp106Thr
	Gln103Ser + Trp106Pro
	Gly102Asp + Gln103Ser
	Gly102Ser + Trp106Gln
	Ser101Asp + Gly102Pro
15	Leu 96Cys + Trp106Asp
	Asp 99Glu + Gly102Ser
	Gly102Asp + Trp106Val
	Gly 97Ser + Trp106Phe
	Gln103Asp + Tyr104Thr
20	Ala 98His + Gly100Gln
	Ser105Glu + Trp106Leu
	Leu 96His + Tyr104Thr
	Gly 97Pro + Ser101Glu
	Val 95Thr + Trp106Ile
25	Gly100Asp + Tyr104Ile
	Val 95Pro + Gln103Asn
	Gln103Asn + Trp106Ile
	Ala 98His + Gly102Pro
	Trp106Asn + Ile107His
30	Val 95Gln + Leu 96Asp
	Gly 97Asp + Ala 98Gln
	Gly100Ser + Ser101Glu
	Val 95Asp + Tyr104Gly
	Tyr104Ala + Ser105Asp
35	Gly100Pro + Ser105Glu
	Leu 96Cys + Tyr104Leu
	Val 95Gly + Gly100Ser
	Gly102Gln + Tyr104Ser
	Ala 98Gly + Trp106Phe
40	Gly100Asp + Trp106Phe
	Val 95Glu + Ala 98Gln
	Ser101Glu + Tyr104Asn
	Leu 96Val + Ser101Asp
	Gly102Glu + Gln103Asn
45	Gly102Glu + Trp106Gly
	Ala 98Gln + Gly100Asp
	Gly100Gln + Gln103Ser
	Gly 97Glu + Tyr104Leu
	Ser101Asp + Gly102Ser
50	Ala 98His + Ser101Asp

Gly 97Asp + Gln103Asn

TABLE 8

Loop 2 - Triple Mutation Variants	
5	Val 95Gln + Leu 96Thr + Ser101Glu Ala 98His + Gln103Glu + Trp106Cys Ala 98Gln + Ser101Glu + Tyr104Met Ser101Asp + Gln103Ser + Ile107Cys Ala 98Pro + Asp 99Glu + Gly102Pro
10	Val 95Pro + Gly 97Glu + Gly100Gln Ser101Glu + Gly102Pro + Ile107His Leu 96Pro + Gly100Pro + Gly102Asn Gly100Glu + Gly102Asn + Trp106Tyr Ala 98Asn + Gln103Glu + Ile107Ser
15	Gly 97Pro + Gly100Asp + Trp106Met Gln103Asn + Tyr104Leu + Ser105Asp Gly 97Pro + Ala 98Gln + Tyr104Cys Ala 98Gly + Gly100Glu + Gln103Ser Leu 96Ile + Gly 97Pro + Ser105Asp
20	Ala 98Pro + Gly100Pro + Ile107Ala Val 95Pro + Gln103Asp + Ile107Met Val 95Gln + Ser101Glu + Trp106Phe Leu 96Val + Ser101Glu + Ile107Pro Leu 96Gly + Gly 97Glu + Trp106Thr
25	Gly 97Asp + Tyr104Ser + Trp106His Gly 97Ser + Gly100Pro + Tyr104Cys Gln103Ser + Ser105Asp + Ile107His Ala 98Glu + Tyr104Cys + Trp106Phe Val 95Gln + Gly100Pro + Gly102Ser
30	Val 95Ala + Gly102Asp + Tyr104Ser Val 95Ala + Leu 96Met + Ser105Asp Gly102Gln + Trp106Leu + Ile107Gly Leu 96Asn + Gly 97Glu + Ile107Pro Gly100Pro + Gly102Gln + Gln103Glu
35	Gly 97Asp + Ala 98Asn + Trp106Leu Ala 98Gln + Gly100Pro + Trp106His Leu 96Thr + Gly100Asn + Ser105Glu Val 95Ser + Leu 96Asn + Gly 97Pro Gly100Gln + Ser105Glu + Trp106Gln
40	Gly 97Glu + Tyr104Thr + Trp106Val Leu 96Ala + Ala 98Gln + Gly100Glu Val 95His + Gly 97Gln + Ser101Glu Val 95Pro + Gly102Asn + Gln103Glu Gln103Asn + Trp106Ile + Ile107Ala
45	Gly 97Ser + Ala 98Glu + Tyr104Gln Val 95Glu + Leu 96Ile + Ile107Gln Leu 96Gln + Ala 98Ser + Asp 99Glu Leu 96Pro + Ser101Glu + Gly102Pro Gly 97Asn + Ala 98Pro + Gly100Pro

	Gly 97Asn + Ala 98Glu + Gly100Asn
	Gly102Pro + Trp106Ala + Ile107Pro
	Gly100Ser + Gly102Glu + Trp106Cys
5	Leu 96Thr + Gly102Glu + Ile107Val
	Leu 96Cys + Trp106Leu + Ile107Pro
	Leu 96Thr + Ser105Glu + Trp106Tyr
	Leu 96Ala + Gly100Asp + Ser101Asp
	Gly 97Asn + Ser101Glu + Gly102Asp
10	Val 95Gln + Ser101Asp + Gly102Asp
	Asp 99Glu + Gly100Asp + Trp106Phe
	Tyr104Glu + Ser105Asp + Ile107Asp
	Leu 96Glu + Ser101Glu + Trp106Val
	Tyr104Met + Ser105Asp + Ile107Asp
15	Gly 97Asp + Gly100Asp + Trp106Pro
	Val 95Ala + Gly 97Asp + Asp 99Glu

TABLE 9

Loop 2 - Quadruple Mutation Variants	
20	Leu 96Gln + Gly 97Ser + Ser101Glu + Trp106Val
	Val 95Ala + Ala 98Gln + Gly100Asn + Gln103Asp
	Val 95Gln + Tyr104Ile + Trp106Gly + Ile107Pro
	Val 95Met + Leu 96Gly + Gly100Pro + Trp106Gly
	Ala 98Gln + Gly100Pro + Tyr104Thr + Trp106His
25	Gly 97Pro + Ala 98His + Gly100Pro + Ile107Asp
	Ala 98Pro + Gly100Glu + Trp106Ser + Ile107Met
	Leu 96Gln + Gly 97Ser + Ser105Asp + Ile107Val
	Ala 98Gly + Ser101Asp + Trp106Ala + Ile107Gln
	Val 95Ser + Gly 97Ser + Asp 99Glu + Gln103Ser
30	Leu 96Thr + Gly 97Ser + Asp 99Glu + Tyr104Asn
	Val 95Thr + Leu 96Gln + Ala 98Pro + Ser105Glu
	Val 95Gly + Gly 97Ser + Tyr104Asn + Trp106Glu
	Leu 96Gln + Gly 97Ser + Tyr104Thr + Ile107Glu
	Val 95Ser + Leu 96Pro + Gly100Gln + Ser101Asp
	Leu 96Met + Gly100Ser + Ser101Asp + Trp106Asn
35	Leu 96Ile + Ala 98Ser + Gly100Pro + Gly102Glu
	Val 95Asn + Ala 98Gly + Gln103Ser + Tyr104Val
	Gly 97Asn + Asp 99Glu + Gly102Asn + Trp106His
	Gly 97Ser + Gly102Asp + Gln103Asp + Ile107His
	Val 95Pro + Gly100Glu + Ser101Glu + Tyr104Gly
40	Ala 98Pro + Gly100Asp + Ser101Asp + Ile107Cys
	Leu 96Gly + Ser101Asp + Gly102Asp + Ile107Gly
	Val 95His + Tyr104Asp + Ser105Asp + Trp106Ala
	Gly102Pro + Ser105Asp + Trp106Asp + Ile107Thr
	Leu 96Glu + Ala 98Gln + Gly102Asp + Tyr104Pro
45	Ala 98Thr + Asp 99Glu + Gly100Glu + Ser101Glu
	Gly 97Ser + Ala 98Glu + Asp 99Glu + Gly100Glu
	Leu 96Asp + Gly 97Glu + Gly100Glu + Ile107Asn
	Leu 96Asn + Gly100Asp + Ser101Asp + Gly102Glu
	Val 95Gly + Ser101Glu + Gly102Asp + Gln103Asp

	Val 95His + Leu 96Glu + Gly100Gln + Ser101Glu
	Leu 96Glu + Gly100Gln + Ser101Asp + Gly102Ser
	Gly 97Asp + Gly100Asp + Gly102Pro + Ile107Gly
	Gly 97Glu + Asp 99Glu + Gly100Pro + Tyr104Ser
5	Leu 96Ile + Gly 97Gln + Gln103Glu + Ser105Glu
	Gln103Asp + Ser105Asp + Trp106Asn + Ile107His
	Val 95Pro + Ala 98Pro + Gln103Glu + Ser105Asp
	Val 95His + Asp 99Glu + Ser101Glu + Gly102Pro
10	Leu 96Asn + Asp 99Glu + Gly100Asn + Ser101Glu
	Ala 98Asp + Asp 99Glu + Ser101Asp + Ile107Pro
	Leu 96Thr + Gly 97Glu + Gly100Glu + Gly102Asp
	Val 95Glu + Gly102Asp + Tyr104Ser + Ile107Glu
	Leu 96Gly + Gly102Asp + Gln103Asp + Ser105Glu
	Gly102Glu + Gln103Glu + Ser105Glu + Trp106Cys
15	Asp 99Glu + Ser101Glu + Gly102Glu + Gln103Asn
	Asp 99Glu + Ser101Glu + Gly102Glu + Trp106Gly
	Gly102Glu + Gln103Asn + Tyr104Asp + Ile107Thr
	Val 95His + Leu 96Val + Gln103Glu + Ile107Glu
	Gly 97Ser + Gly102Ser + Gln103Glu + Ile107Glu
20	Val 95Glu + Leu 96Asp + Gln103Asp + Ile107Asn
	Val 95Thr + Gly102Glu + Trp106Tyr + Ile107Asp
	Val 95Glu + Gly 97Glu + Ala 98Gly + Gly100Asp
	Leu 96Ala + Gly 97Pro + Ala 98Asp + Ser101Asp
	Val 95Asp + Leu 96Asp + Tyr104Glu + Ile107Ser
25	Val 95Pro + Gly102Glu + Tyr104Pro + Ser105Asp
	Leu 96Asn + Gly102Asp + Gln103Asn + Ser105Glu
	Leu 96Asn + Gly102Asp + Tyr104Ala + Ser105Glu
	Leu 96Ser + Gly 97Gln + Gly102Glu + Ser105Asp
	Leu 96Thr + Asp 99Glu + Gly102Asp + Ile107Gly

30

TABLE 10

## Loop 3 - Single Mutation Variants

	Leu126Ala
	Leu126Asn
35	Leu126Asp
	Leu126Cys
	Leu126Gln
	Leu126Glu
	Leu126Gly
40	Leu126His
	Leu126Ile
	Leu126Met
	Leu126Pro
	Leu126Ser
45	Leu126Thr
	Leu126Val
	Gly127Asn
	Gly127Asp
	Gly127Gln

	Gly127Glu
	Gly127Pro
	Gly127Ser
	Gly128Asn
5	Gly128Asp
	Gly128Gln
	Gly128Glu
	Gly128Pro
	Gly128Ser
10	Pro129Asn
	Pro129Asp
	Pro129Gln
	Pro129Glu
	Pro129Gly
15	Pro129Ser
	Ser130Asp
	Ser130Glu
	Gly131Asn
	Gly131Asp
20	Gly131Gln
	Gly131Glu
	Gly131Pro
	Gly131Ser
	Ser132Asp
25	Ser132Glu
	Ala133Asn
	Ala133Asp
	Ala133Gln
	Ala133Glu
30	Ala133Gly
	Ala133His
	Ala133Pro
	Ala133Ser
	Ala133Thr

35

TABLE 11

	<b>Loop 3 - Double Mutation Variants</b>
	Leu126Gln + Ser130Glu
	Gly131Gln + Ala133Asn
40	Pro129Asp + Gly131Gln
	Gly128Ser + Ser130Glu
	Leu126Pro + Ala133Gly
	Gly127Asp + Ala133Gly
	Leu126Asp + Pro129Gln
45	Gly131Asn + Ala133Gln
	Gly127Pro + Gly131Glu
	Gly128Asn + Gly131Asp
	Pro129Gln + Ser130Glu
	Gly128Pro + Ser130Asp

	Gly128Gln + Pro129Ser
	Gly128Asn + Pro129Gly
	Leu126Val + Ser130Asp
	Leu126Val + Pro129Ser
5	Leu126Cys + Pro129Glu
	Gly127Asp + Ala133Thr
	Gly128Pro + Pro129Glu
	Gly127Ser + Gly131Asp
	Leu126His + Pro129Asp
10	Gly131Pro + Ala133Glu
	Gly127Ser + Gly128Ser
	Pro129Asn + Gly131Glu
	Leu126Val + Pro129Asp
	Pro129Gly + Ala133Asp
15	Leu126Val + Ser130Glu
	Pro129Glu + Ala133Pro
	Pro129Gly + Ser130Asp
	Leu126His + Gly128Glu
	Gly128Asn + Ser132Glu
20	Gly127Pro + Ser132Asp
	Gly127Gln + Pro129Gln
	Gly128Pro + Pro129Asp
	Gly128Asn + Ser130Glu
	Leu126Cys + Pro129Asn
25	Pro129Asn + Ser132Glu
	Leu126Ser + Ser132Asp
	Gly128Glu + Gly131Ser
	Pro129Asn + Ser130Asp
	Leu126Ser + Ser132Glu
30	Pro129Gln + Gly131Pro
	Gly127Asp + Gly128Gln
	Gly128Gln + Pro129Glu
	Gly127Pro + Pro129Gly
	Pro129Gln + Ala133Gln
35	Leu126Val + Gly128Asp
	Gly128Ser + Ser132Glu
	Leu126Asn + Pro129Gly
	Leu126Ile + Ala133Gly
	Gly128Ser + Gly131Gln
40	Gly127Ser + Ser130Asp
	Leu126Cys + Ser132Asp
	Gly127Pro + Ser130Glu
	Leu126His + Ala133Asp
	Gly131Ser + Ala133Glu
45	Gly131Pro + Ala133Gln
	Gly131Asp + Ala133Ser
	Leu126Asp + Ala133Asn
	Leu126Glu + Pro129Gln

---



TABLE 12

Loop 3 - Triple Mutation Variants		
5	Leu126His + Pro129Glu + Ala133Asn	
	Leu126Asp + Gly128Ser + Gly131Gln	
	Pro129Asn + Gly131Ser + Ser132Glu	
	Gly128Pro + Pro129Asn + Ser130Glu	
	Gly128Gln + Ser130Glu + Ala133Ser	
10	Gly131Gln + Ser132Glu + Ala133Gln	
	Gly128Asp + Gly131Ser + Ala133Asn	
	Gly131Ser + Ser132Asp + Ala133Pro	
	Pro129Ser + Gly131Gln + Ala133Glu	
	Gly128Asn + Ser130Glu + Gly131Gln	
15	Leu126Gly + Gly127Gln + Gly131Pro	
	Leu126Pro + Gly127Glu + Gly128Pro	
	Leu126Ser + Pro129Ser + Ser132Asp	
	Gly128Ser + Ser132Glu + Ala133Asn	
	Leu126Val + Ser132Glu + Ala133Gln	
20	Pro129Gly + Ser130Glu + Gly131Pro	
	Leu126Thr + Gly127Pro + Ala133Asn	
	Leu126His + Ser130Asp + Ala133Pro	
	Leu126Cys + Gly127Ser + Pro129Ser	
	Leu126Gly + Ser132Asp + Ala133Ser	
25	Gly128Gln + Pro129Gln + Gly131Asn	
	Gly128Asp + Gly131Asn + Ala133His	
	Leu126Cys + Ser130Glu + Ala133Gly	
	Gly127Ser + Ser130Asp + Ala133Gly	
	Leu126His + Pro129Asn + Ser130Asp	
30	Leu126Asn + Gly131Asp + Ala133Gln	
	Leu126Met + Gly128Asn + Ser132Asp	
	Leu126Glu + Gly127Gln + Ala133His	
	Leu126Met + Ser132Asp + Ala133His	
	Ser130Glu + Gly131Gln + Ala133Gln	
35	Gly127Pro + Gly128Ser + Ala133Ser	
	Leu126Ala + Pro129Gly + Ser132Glu	
	Gly131Asn + Ser132Asp + Ala133Asn	
	Leu126Val + Gly131Asp + Ala133Ser	
	Leu126Ser + Gly127Asn + Ala133Gln	
40	Pro129Gln + Ser130Glu + Ala133His	
	Leu126Met + Gly127Ser + Ser130Asp	
	Leu126Cys + Pro129Asn + Gly131Asp	
	Pro129Ser + Ser130Asp + Ala133Asn	
	Leu126Ser + Pro129Gly + Ser132Glu	
45	Gly127Ser + Pro129Gln + Ser132Asp	
	Gly127Pro + Gly128Asn + Pro129Gln	
	Leu126His + Ser132Asp + Ala133Asn	
	Gly128Pro + Pro129Glu + Ala133Thr	
	Pro129Ser + Gly131Glu + Ala133Pro	
	Leu126His + Gly128Pro + Pro129Gln	

	Leu126Met + Gly127Asp + Gly128Asp
	Gly128Pro + Gly131Glu + Ser132Asp
	Gly131Asp + Ser132Glu + Ala133Pro
	Gly128Glu + Pro129Glu + Ala133Asn
5	Pro129Ser + Ser132Glu + Ala133Glu
	Leu126Asn + Ser130Glu + Gly131Asp
	Pro129Asn + Ser130Glu + Gly131Asp
	Leu126His + Ser130Glu + Gly131Glu
	Pro129Glu + Ser130Asp + Gly131Asn
10	Gly127Ser + Pro129Asp + Ser130Asp
	Ser130Asp + Gly131Asp + Ser132Asp
	Gly128Asp + Ser130Glu + Gly131Asn
	Leu126Met + Gly128Glu + Ser130Asp
	Gly128Asp + Pro129Asn + Ser130Glu

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TABLE 13

## Loop 3 - Quadruple Mutation Variants

	Leu126Ser + Pro129Asn + Ser130Asp + Ala133His
	Leu126Met + Pro129Ser + Ser132Glu + Ala133Asn
20	Gly127Ser + Gly131Gln + Ser132Glu + Ala133Gln
	Leu126Asn + Gly127Pro + Gly128Glu + Pro129Gly
	Leu126Asn + Pro129Gly + Gly131Asp + Ala133Gly
	Leu126Gly + Pro129Gly + Ser132Glu + Ala133Pro
	Leu126Gly + Gly127Asp + Pro129Gly + Gly131Pro
25	Gly127Asn + Pro129Gln + Gly131Asp + Ala133Gly
	Leu126Pro + Gly127Ser + Gly128Gln + Ser130Glu
	Leu126Ala + Gly127Gln + Pro129Asn + Ser130Glu
	Leu126Asn + Gly127Ser + Ser130Glu + Ala133Thr
	Gly128Gln + Pro129Gln + Ser130Asp + Gly131Ser
30	Leu126His + Gly128Ser + Gly131Ser + Ser132Asp
	Leu126Gln + Pro129Ser + Ser130Asp + Ala133His
	Leu126Val + Gly128Pro + Pro129Asn + Ala133Asp
	Leu126Val + Pro129Gly + Ser130Glu + Ala133Thr
	Leu126Thr + Gly127Pro + Ser132Glu + Ala133Thr
35	Gly128Asp + Pro129Gly + Gly131Pro + Ala133Ser
	Leu126Asn + Gly128Glu + Pro129Gln + Gly131Pro
	Leu126Pro + Gly127Pro + Pro129Ser + Ser130Asp
	Gly127Pro + Gly128Gln + Gly131Glu + Ser132Glu
	Leu126Ile + Gly127Gln + Gly131Asp + Ser132Glu
40	Leu126Val + Gly131Asp + Ser132Asp + Ala133Pro
	Gly128Asp + Pro129Asp + Gly131Asn + Ala133Pro
	Pro129Asn + Gly131Ser + Ser132Asp + Ala133Asp
	Leu126Gln + Gly131Pro + Ser132Asp + Ala133Asp
	Gly127Pro + Ser130Glu + Gly131Glu + Ala133His
45	Leu126Gln + Pro129Gln + Ser130Asp + Gly131Glu
	Gly127Ser + Ser130Asp + Gly131Glu + Ala133Gln
	Leu126Ser + Gly127Pro + Pro129Glu + Ser130Glu
	Ser130Glu + Gly131Glu + Ser132Glu + Ala133Ser
	Gly127Gln + Ser130Glu + Gly131Asp + Ser132Asp

	Gly128Gln + Ser130Glu + Gly131Asp + Ser132Asp
	Gly127Asn + Ser130Glu + Gly131Asp + Ser132Asp
	Gly127Ser + Pro129Asp + Ser130Glu + Gly131Glu
5	Gly127Asn + Pro129Asp + Ser130Asp + Gly131Asp
	Gly128Asn + Pro129Glu + Ser130Glu + Gly131Asp
	Leu126Ser + Gly128Asp + Ser130Glu + Ala133Pro
	Gly127Asn + Gly128Asp + Ser130Glu + Ala133Pro
	Gly128Glu + Ser130Glu + Gly131Pro + Ala133His
10	Leu126Val + Ser130Asp + Ser132Asp + Ala133Asn
	Pro129Ser + Ser130Glu + Ser132Asp + Ala133Gly
	Leu126His + Ser130Glu + Ser132Asp + Ala133His
	Leu126Ala + Ser130Glu + Ser132Glu + Ala133Asn
	Gly127Pro + Gly128Gln + Ser130Asp + Ser132Glu
15	Leu126Ser + Ser130Asp + Gly131Pro + Ser132Asp
	Ser130Glu + Gly131Pro + Ser132Glu + Ala133Ser
	Gly128Gln + Ser130Asp + Gly131Ser + Ser132Glu
	Leu126Ala + Pro129Asn + Ser130Asp + Ser132Glu
	Gly127Gln + Gly128Pro + Pro129Glu + Gly131Asp
20	Gly128Gln + Pro129Asp + Gly131Glu + Ala133Asn
	Leu126Asn + Pro129Glu + Gly131Asp + Ala133Ser
	Leu126Met + Pro129Glu + Gly131Glu + Ala133Thr
	Gly127Asp + Gly128Gln + Pro129Asp + Ala133Gln
	Leu126His + Pro129Gly + Gly131Glu + Ala133Glu
	Gly128Glu + Pro129Gly + Gly131Asp + Ala133Asn
25	Pro129Gly + Ser130Glu + Ser132Asp + Ala133Glu
	Leu126Gln + Ser130Glu + Ser132Glu + Ala133Glu
	Leu126Gly + Pro129Asp + Ser130Glu + Ser132Glu
	Pro129Asp + Ser130Glu + Gly131Ser + Ser132Asp

TABLE 14

## Loop 4 - Single Mutation Variants

	Gly154Asn
	Gly154Asp
	Gly154Gln
35	Gly154Glu
	Gly154Pro
	Gly154Ser
	Asn155Asp
	Asn155Gln
40	Asn155Glu
	Asn155Ser
	Glu156Asp
	Gly157Asn
	Gly157Asp
45	Gly157Gln
	Gly157Glu
	Gly157Pro
	Gly157Ser
	Thr158Asn

	Thr158Asp
	Thr158Gln
	Thr158Glu
5	Thr158Gly
	Thr158Pro
	Thr158Ser
	Ser159Asp
	Ser159Glu
10	Gly160Asn
	Gly160Asp
	Gly160Gln
	Gly160Glu
	Gly160Pro
	Gly160Ser
15	Ser161Asp
	Ser161Glu
	Ser162Asp
	Ser162Glu
20	Ser163Asp
	Ser163Glu
	Thr164Asn
	Thr164Asp
	Thr164Gln
25	Thr164Glu
	Thr164Gly
	Thr164Pro
	Thr164Ser
	Val165Ala
30	Val165Asn
	Val165Asp
	Val165Cys
	Val165Gln
	Val165Glu
35	Val165Gly
	Val165His
	Val165Met
	Val165Pro
	Val165Ser
40	Val165Thr
	Gly166Asn
	Gly166Asp
	Gly166Gln
	Gly166Glu
45	Gly166Pro
	Gly166Ser
	Tyr167Ala
	Tyr167Asn
	Tyr167Asp
50	Tyr167Cys
	Tyr167Gln

	Tyr167Glu
	Tyr167Gly
	Tyr167His
	Tyr167Ile
5	Tyr167Leu
	Tyr167Met
	Tyr167Pro
	Tyr167Ser
	Tyr167Thr
10	Tyr167Val

TABLE 15

Loop 4 - Double Mutation Variants	
15	Asn155Ser + Glu156Asp
	Gly154Ser + Tyr167Gln
	Gly154Glu + Val165Ala
	Asn155Glu + Thr164Pro
	Gly157Pro + Ser159Asp
	Gly154Ser + Ser161Asp
20	Ser161Glu + Val165Pro
	Gly154Gln + Ser161Glu
	Asn155Asp + Thr158Pro
	Thr164Asn + Gly166Gln
	Asn155Glu + Tyr167His
25	Glu156Asp + Thr158Gly
	Gly154Pro + Gly157Glu
	Asn155Ser + Tyr167Asp
	Thr158Pro + Gly166Asp
	Thr164Gln + Tyr167Glu
30	Gly157Gln + Thr158Glu
	Thr158Asn + Ser162Asp
	Gly154Asn + Tyr167Glu
	Gly157Gln + Ser161Asp
	Thr164Asp + Tyr167Ala
35	Gly160Asp + Val165His
	Gly154Glu + Gly157Ser
	Glu156Asp + Tyr167Ile
	Asn155Ser + Thr158Asp
	Gly157Gln + Thr164Pro
40	Thr164Ser + Tyr167Ile
	Ser159Glu + Tyr167Thr
	Thr164Glu + Val165Gln
	Thr158Gly + Gly160Ser
	Ser161Asp + Gly166Pro
45	Gly154Glu + Gly166Ser
	Gly160Asp + Val165Asn
	Ser162Glu + Val165Gln
	Gly157Asn + Ser159Glu
	Ser161Asp + Val165Asn

	Asn155Asp + Val165Pro
	Glu156Asp + Gly166Ser
	Gly154Pro + Ser159Asp
5	Gly154Ser + Tyr167Cys
	Gly160Pro + Thr164Asp
	Ser161Glu + Val165Gly
	Ser162Glu + Tyr167Asn
	Gly154Asn + Gly166Glu
	Ser161Glu + Tyr167Ala
10	Gly160Gln + Val165Pro
	Gly154Glu + Val165Gly
	Gly160Ser + Ser163Asp
	Gly157Glu + Thr158Asn
	Gly160Asp + Val165Pro
15	Gly160Asn + Ser162Asp
	Thr164Gln + Gly166Gln
	Asn155Ser + Thr158Gln
	Ser161Glu + Tyr167Gly
	Ser162Asp + Gly166Ser
20	Gly154Glu + Thr158Gly
	Gly154Ser + Thr158Ser
	Gly157Asp + Gly160Pro
	Ser163Glu + Val165His
	Gly154Pro + Gly166Asp

25

TABLE 16

## Loop 4 - Triple Mutation Variants

	Gly154Gln + Asn155Ser + Glu156Asp
	Gly154Ser + Gly160Asp + Tyr167Gln
30	Asn155Glu + Gly157Ser + Thr164Pro
	Gly157Asn + Ser159Asp + Gly160Ser
	Glu156Asp + Gly160Ser + Val165Thr
	Gly160Pro + Ser162Glu + Thr164Asn
	Gly154Ser + Glu156Asp + Thr158Gln
35	Gly160Asn + Ser162Glu + Gly166Ser
	Gly160Ser + Val165Gly + Gly166Gln
	Thr158Gln + Ser162Asp + Tyr167Val
	Gly157Gln + Ser162Glu + Tyr167Leu
	Ser162Glu + Thr164Gln + Val165Cys
40	Gly157Ser + Val165Met + Gly166Glu
	Gly154Ser + Glu156Asp + Gly166Pro
	Thr158Ser + Ser161Asp + Thr164Gly
	Glu156Asp + Gly157Ser + Gly160Asn
	Gly154Gln + Asn155Asp + Gly166Ser
45	Ser163Glu + Val165Thr + Tyr167Pro
	Gly157Asp + Thr158Gln + Val165Ser
	Gly157Asn + Ser159Asp + Gly166Ser
	Gly160Gln + Ser163Glu + Val165Met
	Gly154Asn + Asn155Asp + Gly157Pro

	Glu156Asp + Thr158Asn + Val165Cys
	Thr158Asn + Gly160Glu + Thr164Pro
	Gly154Asn + Gly157Pro + Thr158Gln
5	Asn155Glu + Gly157Ser + Thr158Gln
	Thr158Glu + Gly160Ser + Tyr167Val
	Asn155Gln + Glu156Asp + Thr164Ser
	Asn155Ser + Ser162Glu + Val165Met
	Gly154Gln + Thr158Gly + Gly166Asp
10	Ser163Glu + Val165Ala + Gly166Asn
	Asn155Ser + Gly160Glu + Thr164Gln
	Gly157Asp + Thr164Ser + Gly166Pro
	Ser163Asp + Thr164Glu + Tyr167Met
	Ser163Asp + Thr164Asp + Val165Met
15	Glu156Asp + Gly157Asp + Thr164Gln
	Gly157Gln + Gly166Asp + Tyr167Glu
	Ser161Asp + Ser162Glu + Tyr167His
	Gly154Asn + Ser159Glu + Ser162Glu
	Ser159Asp + Ser162Glu + Val165Cys
20	Ser159Glu + Gly160Ser + Ser161Asp
	Thr158Asp + Ser161Glu + Ser162Glu
	Ser161Glu + Ser163Asp + Thr164Ser
	Ser161Glu + Ser163Glu + Val165His
	Asn155Glu + Glu156Asp + Thr158Glu
25	Gly157Glu + Thr164Glu + Val165Gly
	Ser161Asp + Ser163Glu + Thr164Glu
	Gly157Glu + Thr158Gln + Ser159Glu
	Gly157Glu + Ser159Asp + Tyr167Cys
	Gly157Asp + Ser163Glu + Thr164Glu
30	Ser159Glu + Ser163Asp + Thr164Gly
	Ser159Asp + Ser163Asp + Thr164Asn
	Thr158Asp + Ser161Asp + Ser163Glu
	Thr158Glu + Ser162Asp + Thr164Asn
	Thr158Glu + Ser162Asp + Val165Thr
35	Gly157Ser + Thr158Asp + Ser162Glu
	Thr158Asp + Ser163Glu + Thr164Asn
	Thr158Glu + Ser163Asp + Tyr167Gly
	Glu156Asp + Gly166Glu + Tyr167Ile
	Asn155Glu + Gly157Pro + Thr164Asp

TABLE 17

## Loop 4 - Quadruple Mutation Variants

	Ser159Glu + Thr164Ser + Val165Thr + Gly166Pro
	Asn155Ser + Gly157Pro + Val165Ser + Gly166Glu
	Gly157Asn + Val165Pro + Gly166Glu + Tyr167Val
45	Thr158Ser + Gly160Gln + Val165His + Gly166Asp
	Gly154Ser + Gly157Pro + Ser163Glu + Thr164Ser
	Gly157Gln + Gly160Asp + Thr164Ser + Val165Asn
	Gly157Asn + Gly160Asp + Val165Cys + Tyr167Leu
	Glu156Asp + Thr158Ser + Val165Asn + Gly166Pro

5      Glu156Asp + Thr158Pro + Thr164Gln + Val165Pro  
Asn155Gln + Glu156Asp + Thr164Gly + Val165Thr  
Thr158Gly + Gly160Ser + Ser163Asp + Tyr167Asn  
Ser159Asp + Gly160Gln + Gly166Ser + Tyr167Pro  
10      Gly154Pro + Thr164Gln + Val165Gly + Gly166Asp  
Gly154Asn + Gly160Pro + Ser161Glu + Gly166Pro  
Asn155Ser + Gly157Asn + Thr164Gln + Tyr167Asp  
Gly157Asn + Thr158Asn + Ser163Glu + Val165Gln  
Gly160Glu + Ser161Asp + Val165Met + Tyr167Pro  
15      Asn155Glu + Glu156Asp + Thr158Gln + Gly166Pro  
Asn155Asp + Glu156Asp + Val165Asn + Gly166Asn  
Asn155Asp + Glu156Asp + Gly160Ser + Thr164Asn  
Gly154Ser + Thr158Gln + Ser162Glu + Ser163Glu  
Gly154Asn + Asn155Gln + Ser163Glu + Thr164Glu  
20      Glu156Asp + Gly157Glu + Gly160Gln + Thr164Gly  
Glu156Asp + Gly157Glu + Thr158Ser + Val165Cys  
Gly154Pro + Gly157Pro + Thr158Asp + Ser159Asp  
Gly154Ser + Gly157Asn + Thr158Glu + Ser159Glu  
Gly157Pro + Gly160Pro + Gly166Asp + Tyr167Glu  
25      Gly154Asn + Ser161Glu + Ser162Glu + Tyr167Asn  
Gly154Asp + Asn155Asp + Thr164Gln + Gly166Asn  
Gly154Gln + Ser159Glu + Gly160Glu + Ser161Asp  
Thr158Ser + Ser159Asp + Gly160Asp + Ser161Asp  
Asn155Ser + Glu156Asp + Gly157Asp + Thr158Glu  
30      Gly157Asn + Ser159Asp + Ser161Glu + Ser162Glu  
Gly154Asn + Glu156Asp + Gly157Glu + Thr164Glu  
Gly157Gln + Gly160Asp + Ser162Asp + Val165Thr  
Gly160Glu + Ser162Asp + Thr164Asn + Gly166Gln  
Gly154Asp + Asn155Ser + Glu156Asp + Thr164Ser  
35      Gly154Asp + Glu156Asp + Gly157Glu + Thr158Gly  
Gly154Gln + Gly157Pro + Ser159Asp + Ser161Asp  
Ser159Glu + Ser161Asp + Gly166Ser + Tyr167His  
Ser159Asp + Ser161Asp + Gly166Pro + Tyr167Ser  
Glu156Asp + Thr158Glu + Val165Ala + Gly166Gln  
40      Glu156Asp + Thr158Asp + Gly166Pro + Tyr167Ala  
Asn155Gln + Thr158Asp + Thr164Asp + Tyr167Val  
Ser163Glu + Thr164Asp + Val165Met + Gly166Glu  
Ser161Asp + Ser163Asp + Val165Thr + Tyr167His  
Ser161Asp + Ser163Glu + Thr164Gln + Gly166Asn  
45      Gly157Pro + Ser159Glu + Ser161Asp + Ser163Glu  
Gly154Pro + Glu156Asp + Ser163Asp + Thr164Glu  
Asn155Asp + Glu156Asp + Thr158Asp + Thr164Asn  
Glu156Asp + Ser159Asp + Thr164Asp + Val165Ala  
Thr158Gln + Ser159Asp + Ser163Glu + Val165Cys  
50      Gly154Gln + Ser159Asp + Ser163Asp + Gly166Pro  
Asn155Ser + Gly160Asp + Ser162Glu + Thr164Asp  
Gly154Gln + Gly160Asp + Ser162Glu + Thr164Glu  
Glu156Asp + Gly160Pro + Val165Pro + Gly166Glu  
Gly160Glu + Ser163Asp + Thr164Gly + Tyr167Leu  
Gly160Glu + Ser163Glu + Thr164Pro + Gly166Gln



Asn155Asp + Thr158Pro + Ser163Glu + Thr164Asp  
 Asn155Ser + Glu156Asp + Ser163Asp + Gly166Glu

TABLE 18

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5                      Loop 5 - Single Mutation Variants

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	Ala187Asn
	Ala187Asp
	Ala187Gln
	Ala187Glu
10	Ala187Gly
	Ala187His
	Ala187Pro
	Ala187Ser
	Ala187Thr
15	Ser188Asp
	Ser188Glu
	Phe189Ala
	Phe189Asn
	Phe189Asp
20	Phe189Cys
	Phe189Gln
	Phe189Glu
	Phe189Gly
	Phe189His
25	Phe189Ile
	Phe189Leu
	Phe189Met
	Phe189Pro
	Phe189Ser
30	Phe189Thr
	Phe189Tyr
	Phe189Val
	Ser190Asp
	Ser190Glu
35	Ser191Asp
	Ser191Glu

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TABLE 19

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Loop 5 - Double Mutation Variants

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40	Ala187Asp + Phe189Gln
	Ala187Ser + Ser188Asp
	Ser188Glu + Phe189Pro
	Ala187Asp + Phe189His
	Ala187Asn + Ser191Glu
45	Ala187Gln + Ser191Asp
	Ala187Glu + Phe189Pro
	Ala187Pro + Phe189Asp

	Ser188Asp + Phe189Cys
	Phe189His + Ser191Asp
	Ser188Glu + Phe189Ala
5	Ala187His + Ser188Asp
	Ala187Asn + Ser188Glu
	Ser188Glu + Phe189Gln
	Ala187Asp + Phe189Ser
	Ser188Asp + Phe189Val
10	Ala187Gln + Ser188Glu
	Ala187Ser + Ser188Glu
	Ala187Pro + Ser191Asp
	Ser188Glu + Phe189Val
	Phe189Ser + Ser191Glu
	Ala187Gly + Ser191Glu
15	Ala187Asn + Ser191Asp
	Ala187Thr + Ser191Asp
	Ala187His + Ser188Glu
	Ser188Glu + Phe189Gly
	Ala187Ser + Phe189Ile
20	Ser188Glu + Phe189Met
	Phe189Asn + Ser191Asp
	Ala187Gln + Phe189Tyr
	Ala187Gln + Ser191Glu
	Ala187Ser + Phe189Ala
25	Phe189Val + Ser191Asp
	Ser188Glu + Phe189Leu
	Ala187Pro + Ser188Glu
	Phe189Asn + Ser191Glu
	Phe189Ile + Ser191Asp
30	Ala187Glu + Phe189Met
	Ala187His + Ser191Glu
	Ser188Asp + Phe189Tyr
	Ala187Gly + Phe189Val
	Ser188Asp + Phe189Gln
35	Ala187Gly + Phe189Tyr
	Ala187Gln + Phe189Asp
	Phe189Tyr + Ser191Glu
	Ala187Ser + Ser191Asp
	Ala187Thr + Ser188Glu
40	Ala187Asn + Ser188Asp
	Ala187Gly + Ser188Asp
	Ala187Gly + Phe189Cys
	Phe189Cys + Ser191Glu
	Ala187Asp + Phe189Gly
45	Ser188Asp + Phe189Leu
	Ser188Asp + Phe189Gly
	Ala187Asn + Phe189Asp
	Ala187Pro + Ser191Glu
	Phe189Met + Ser191Asp
50	Ala187Thr + Ser188Asp

Phe189Ala + Ser191Glu  
Phe189Leu + Ser191Glu

TABLE 20

## Loop 5 - Triple Mutation Variants

5	Ala187Pro + Phe189Cys + Ser191Glu
	Ala187Thr + Phe189Tyr + Ser191Glu
	Ala187Ser + Ser188Glu + Phe189Ser
	Ala187Gln + Phe189Asn + Ser191Glu
	Ala187Gln + Ser188Asp + Phe189His
10	Ala187Gln + Ser188Glu + Phe189His
	Ala187Gly + Ser188Asp + Phe189Met
	Ala187Gly + Ser188Asp + Phe189Cys
	Ala187Pro + Phe189His + Ser191Glu
	Ala187Pro + Phe189Gln + Ser191Glu
15	Ala187Asn + Ser188Asp + Phe189Asn
	Ala187Gly + Ser188Glu + Phe189Ser
	Ala187Gln + Phe189Met + Ser191Asp
	Ala187Gly + Ser188Asp + Phe189Pro
	Ala187Thr + Phe189His + Ser191Asp
20	Ala187Asn + Ser188Glu + Phe189Cys
	Ala187Gln + Phe189Val + Ser191Glu
	Ala187Pro + Phe189Met + Ser191Glu
	Ala187Ser + Ser188Glu + Phe189His
	Ala187Ser + Phe189Gln + Ser191Asp
25	Ala187Gln + Ser188Asp + Phe189Pro
	Ala187Gly + Ser188Asp + Phe189Gly
	Ala187His + Phe189Gln + Ser191Glu
	Ala187Thr + Ser188Glu + Phe189Ile
	Ala187Pro + Phe189Gly + Ser191Glu
30	Ala187Thr + Phe189Met + Ser191Glu
	Ala187Gly + Phe189Thr + Ser191Glu
	Ala187Gln + Phe189Leu + Ser191Glu
	Ala187Thr + Phe189Thr + Ser191Asp
	Ala187Gln + Ser188Asp + Phe189Met
35	Ala187Pro + Phe189Ser + Ser191Glu
	Ala187Asp + Ser188Glu + Phe189Val
	Ala187Glu + Ser188Glu + Phe189Ser
	Ala187Asp + Ser188Glu + Phe189Met
	Ala187Asp + Ser188Asp + Phe189Gln
40	Ala187Asp + Ser188Glu + Phe189Cys
	Ala187Asp + Ser188Glu + Phe189Tyr
	Ala187Glu + Ser188Glu + Phe189Tyr
	Ala187Asp + Ser188Asp + Phe189Gly
	Ala187Glu + Ser188Glu + Phe189Leu
45	Ala187Asp + Ser188Glu + Phe189Ser
	Ala187Glu + Ser188Asp + Phe189Gly
	Ala187Asp + Ser188Asp + Phe189Pro
	Ala187Asp + Ser188Glu + Phe189His
	Ala187Glu + Ser188Glu + Phe189Thr

	Ala187Glu + Ser188Asp + Phe189Ile
	Ala187Glu + Ser188Asp + Phe189Asn
	Ala187Ser + Ser188Glu + Phe189Glu
	Ala187Gly + Ser188Asp + Phe189Glu
5	Ala187Gly + Ser188Glu + Phe189Asp
	Ala187Pro + Ser188Glu + Phe189Asp
	Ala187Asp + Ser188Glu + Phe189Glu
	Ala187Glu + Ser188Asp + Phe189Asp
	Ala187Asp + Ser188Glu + Phe189Asp
10	Ala187Glu + Ser188Glu + Phe189Glu
	Ala187Gly + Phe189Glu + Ser191Asp
	Ala187Gly + Phe189Glu + Ser191Glu
	Ala187Thr + Phe189Glu + Ser191Glu
	Ser188Glu + Phe189Glu + Ser191Glu
15	Ser188Glu + Phe189Glu + Ser191Asp

TABLE 21

Loop 5 - Quadruple Mutation Variants	
20	Ala187Ser + Ser188Glu + Phe189Asp + Ser191Asp
	Ala187Pro + Ser188Glu + Phe189Glu + Ser191Glu
	Ala187His + Ser188Glu + Phe189Asp + Ser191Glu
	Ala187Gly + Ser188Asp + Phe189Asp + Ser191Glu
	Ala187His + Ser188Glu + Phe189Glu + Ser191Asp
	Ala187Thr + Ser188Asp + Phe189Asp + Ser191Glu
25	Ala187Asn + Ser188Glu + Phe189Glu + Ser191Glu
	Ala187Pro + Ser188Asp + Phe189Glu + Ser191Glu
	Ala187Pro + Ser188Asp + Phe189Asp + Ser191Asp
	Ala187Ser + Ser188Glu + Phe189Asp + Ser191Glu
	Ala187His + Ser188Asp + Phe189Glu + Ser191Asp
30	Ala187Thr + Ser188Glu + Phe189Asp + Ser191Asp
	Ala187Asn + Ser188Asp + Phe189Glu + Ser191Glu
	Ala187Gln + Ser188Glu + Phe189Asp + Ser191Glu
	Ala187Gly + Ser188Asp + Phe189Glu + Ser191Glu
	Ala187Glu + Ser188Asp + Phe189Gly + Ser191Asp
35	Ala187Glu + Ser188Glu + Phe189Met + Ser191Asp
	Ala187Asp + Ser188Asp + Phe189Ile + Ser191Glu
	Ala187Asp + Ser188Glu + Phe189Leu + Ser191Asp
	Ala187Asp + Ser188Glu + Phe189Thr + Ser191Asp
	Ala187Glu + Ser188Glu + Phe189Leu + Ser191Asp
40	Ala187Glu + Ser188Asp + Phe189Tyr + Ser191Asp
	Ala187Glu + Ser188Glu + Phe189Gln + Ser191Asp
	Ala187Glu + Ser188Glu + Phe189Cys + Ser191Glu
	Ala187Glu + Ser188Glu + Phe189Gln + Ser191Glu
	Ala187Glu + Ser188Glu + Phe189Pro + Ser191Glu
45	Ala187Asp + Ser188Glu + Phe189Ser + Ser191Glu
	Ala187Glu + Ser188Glu + Phe189Cys + Ser191Asp
	Ala187Asp + Ser188Asp + Phe189Leu + Ser191Asp
	Ala187Glu + Ser188Asp + Phe189Ile + Ser191Asp
	Ala187Asp + Ser188Asp + Phe189His + Ser191Glu

	Ala187Glu + Ser188Asp + Phe189His + Ser191Asp
	Ala187Glu + Ser188Asp + Phe189Val + Ser191Asp
	Ala187Asp + Ser188Glu + Phe189Gly + Ser191Glu
	Ala187Asp + Ser188Asp + Phe189Cys + Ser191Asp
5	Ala187Glu + Ser188Glu + Phe189Asn + Ser191Glu
	Ala187Asp + Ser188Asp + Phe189Thr + Ser191Glu
	Ala187Asp + Ser188Asp + Phe189Ile + Ser191Asp
	Ala187Asp + Ser188Asp + Phe189Ala + Ser191Glu
	Ala187Asp + Ser188Asp + Phe189Val + Ser191Glu
10	Ala187Glu + Ser188Glu + Phe189Ala + Ser191Glu
	Ala187Asp + Ser188Asp + Phe189Ser + Ser191Asp
	Ala187Glu + Ser188Asp + Phe189Asn + Ser191Asp
	Ala187Asp + Ser188Asp + Phe189Cys + Ser191Glu
	Ala187Asp + Ser188Glu + Phe189Cys + Ser191Asp
15	Ala187Glu + Ser188Asp + Phe189Ser + Ser191Glu
	Ala187Asp + Ser188Glu + Phe189Tyr + Ser191Glu
	Ala187Asp + Ser188Glu + Phe189Ala + Ser191Asp
	Ala187Gly + Ser188Glu + Phe189Thr + Ser191Asp
	Ala187His + Ser188Asp + Phe189Met + Ser191Glu
20	Ala187Thr + Ser188Asp + Phe189Ser + Ser191Asp
	Ala187Ser + Ser188Glu + Phe189Met + Ser191Asp
	Ala187Ser + Ser188Asp + Phe189Ser + Ser191Asp
	Ala187Thr + Ser188Asp + Phe189Tyr + Ser191Glu
	Ala187Ser + Ser188Glu + Phe189Ala + Ser191Asp
25	Ala187Asn + Ser188Glu + Phe189Gly + Ser191Asp
	Ala187Gln + Ser188Asp + Phe189Asn + Ser191Glu
	Ala187Asn + Ser188Asp + Phe189His + Ser191Glu
	Ala187Gly + Ser188Asp + Phe189Ser + Ser191Glu
	Ala187His + Ser188Asp + Phe189Val + Ser191Asp

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TABLE 22

Multi-loop Double Mutation Variants	
	Leu 96Gly + Ser204Glu
	Gln 59Ser + Asn 62Ser
35	Val 95Gln + Asn218Asp
	Tyr104Cys + Lys213Glu
	Gly127Gln + Ala216Pro
	Ser188Glu + Gly215Asn
	Gly 97Gln + Ile107Ala
40	Gln206Asp + Tyr217Thr
	Asp 60Glu + Gln206Asn
	Thr158Asp + Gln206Ser
	Pro210Gln + Gly215Asn
	Tyr104Glu + Ile107Leu
45	Tyr167Pro + Gly211Glu
	Ile107Leu + Ala187Asp
	Gly 97Glu + Thr164Pro
	Thr 66Pro + Val203Cys
	Ala133Gly + Tyr217Ser

	Ser105Glu + Phe189Val
	Tyr167Asp + Ala187Thr
	Ser161Glu + Ala216Thr
5	Ser 63Asp + Gln103Ser
	Leu 96Gln + Pro129Glu
	Ala 98Gly + Tyr214Glu
	Leu 96Asn + Asn212Ser
	Ser 63Asp + Phe189Leu
10	Thr158Gln + Lys213Glu
	Leu126Gln + Gly160Asp
	Ser159Asp + Tyr217Gln
	Ser101Asp + Val203Ala
	Gly100Asn + Gly215Glu
15	Gln 59Asp + Gly131Gln
	Gly157Glu + Leu209Pro
	Trp106Pro + Tyr217Ile
	Ala216Ser + Gly219Asp
	Thr 66Gln + Leu126Asn
20	Gly102Gln + Gly219Asp
	Asn212Ser + Lys213Asp
	Gln206Ser + Lys213Glu
	Tyr104Glu + Asn155Gln
	Val 95Asp + Leu126Ser
25	Tyr104Asp + Gly166Gln
	Thr 66Pro + Ser204Glu
	Asn 61Glu + Phe189Pro
	Asp 60Glu + Tyr167Ala
	Pro129Gln + Gln206Asp
30	Gly160Asp + Ala216Asn
	Ser161Glu + Gly166Asn
	Leu 96Pro + Gly100Asp
	Trp106Asn + Val203Asn
	Ser101Asp + Gly127Ser
35	Ala133Gln + Val203Asp
	Ser101Asp + Gly202Ser
	Ile107Ala + Gly160Asn
	Ala133Thr + Tyr214Ile
	Phe189Ser + Ser204Asp
40	Gly 97Asp + Trp106Phe
	Gln 59Asn + Glu156Asp
	Pro201Ser + Lys213Glu
	Ser162Glu + Gly202Gln
	Gly 65Ser + Gln206Asp
45	Lys213Asp + Ala216Pro
	Val203Ala + Lys213Asp
	Ala216Thr + Tyr217Pro
	Gly131Asn + Asn218Glu
	Tyr104Glu + Gly131Pro
	Gly127Ser + Thr158Asp
50	Trp106Gly + Ser132Asp

	Asn 62Ser + Ala187Ser
	Ser163Asp + Phe189Ser
	Pro201Gln + Gly215Glu
5	Gly100Gln + Tyr217Thr
	Ser130Glu + Gly154Asn
	Asp 60Glu + Tyr214Thr
	Asn155Glu + Tyr217Gln
	Ala 98Gln + Gly102Asn
10	Pro201Asn + Gly219Asp
	Thr 66Ser + Gly127Gln
	Leu126Glu + Ala216Thr
	Asn 61Ser + Asn155Glu
	Thr 66Ser + Gly157Asp
15	Pro129Ser + Thr164Gln
	Ala216Asp + Tyr217Val
	Ser130Glu + Tyr217Leu
	Asn 62Asp + Tyr214Leu
	Val 95Ser + Phe189Val
20	Gly100Pro + Ser159Asp
	Asn155Gln + Ser204Glu
	Pro129Asp + Val203Ser
	Ser101Glu + Thr158Asn
	Ala187Pro + Asn218Asp
25	Val 95Gly + Ser161Asp
	Gly202Pro + Ala216Gln
	Gly 97Ser + Gly215Asp
	Tyr167Asp + Gln206Ser
	Thr 66Ser + Asn212Glu
30	Ala216Thr + Tyr217Gln
	Ala200Asn + Tyr217Ala
	Asp 60Glu + Val203Pro
	Val 95Thr + Tyr217Met
	Val203Asn + Lys213Glu
35	Gly102Asp + Val203Gly
	Ser130Asp + Ala133Thr
	Tyr104Ala + Gly166Ser
	Leu 96Met + Tyr217Asp
	Ser101Asp + Gly102Pro
40	Ser101Asp + Thr220Pro
	Val 95Asn + Ala216Pro
	Tyr104Asn + Pro129Asp
	Gly202Asn + Gln206Asp
	Gln 59Glu + Ile107Cys
45	Thr 66Glu + Tyr104Pro
	Val 95Met + Asp 99Glu
	Ser204Glu + Gly211Pro
	Pro210Glu + Gly219Ser
	Leu126Pro + Ser204Glu
	Pro129Asp + Ala200His
50	Ile107Gly + Gly215Pro

	Thr 66Glu + Gln206Asn
	Asn155Asp + Leu209His
	Gly211Asp + Tyr217Val
	Ala216Asp + Thr220Gln
5	Thr158Gly + Ser204Asp
	Gly100Glu + Ile107Ser
	Ala 98Ser + Gly154Asn
	Gln103Asn + Ala216Glu
	Gly154Gln + Pro210Gln
10	Leu126Pro + Ala216His
	Ala216His + Tyr217Leu
	Gly154Glu + Tyr217Ser
	Gly 97Ser + Tyr167Thr
	Trp106Ile + Ala216Gly
15	Gly102Ser + Phe189Gly
	Gly154Glu + Gly219Asn
	Lys213Glu + Ala216Pro
	Asn 62Asp + Leu126Ser
	Thr 66Gly + Gln206Glu
20	Gly157Pro + Val203Cys
	Gln 59Asp + Tyr214Ser
	Leu 96Met + Gly100Ser
	Ala 98Gly + Lys213Asp
	Asn 62Gln + Leu 96Asp
25	Gly127Asn + Gln206Glu
	Gly160Pro + Gly219Asn
	Leu 96Thr + Tyr217Ala
	Trp106Phe + Tyr217Thr
	Gly131Pro + Lys213Glu
30	Gly 65Gln + Asp 99Glu
	Gly127Asn + Gly128Gln
	Ala133Asn + Gly154Asn
	Ser204Glu + Gly215Ser
	Glu156Asp + Pro210Ser
35	Asp 60Glu + Gln206Ser
	Asn 61Gln + Ala216Asn
	Pro210Asn + Asn212Asp
	Ala133Asp + Val203Asn
	Gly219Ser + Thr220Gly
40	Ser191Asp + Val203Thr
	Gly160Glu + Ala216Thr
	Ser162Glu + Ala216Gln
	Ala 98Gln + Tyr217Asn
	Val 95Asp + Gln206Asn
45	Tyr104Ser + Ser204Asp
	Gly100Pro + Phe189Gln
	Gly 97Asp + Tyr217His
	Gln206Ser + Gly211Asn
	Ala187Asn + Ser188Asp
50	Ala 98Gly + Asp 99Glu



	Thr164Asn + Phe189Cys
	Val203Gln + Gln206Ser
	Trp106Cys + Gly157Ser
5	Thr158Ser + Gly160Ser
	Ser188Asp + Tyr217Gly
	Gly157Asn + Phe189Met
	Ser188Asp + Ala216Asn
	Gly128Asn + Gly166Ser
10	Leu126Asn + Ala216Ser
	Gly127Asp + Gln206Asn
	Gln 59Glu + Leu 96His
	Ser132Asp + Tyr217Ala
	Gly166Ser + Gly219Glu
15	Ser163Glu + Val203Met
	Ala 98His + Tyr217Met
	Ala 98Pro + Ser130Asp
	Gly160Asn + Ser204Glu
	Gln206Asn + Gly215Asp
20	Gln103Ser + Ser130Asp
	Ala133Gly + Thr220Gly
	Ser132Glu + Ala216Gln
	Asn 61Gln + Ile107His
	Leu126Ala + Gly131Glu
25	Gln206Asp + Thr220Gly
	Gln206Glu + Tyr217Cys
	Gly157Ser + Pro210Asp
	Gly166Glu + Tyr214Gln
	Ser188Glu + Ala216His
30	Thr 66Glu + Gly166Gln
	Gly102Pro + Gly166Glu
	Val 95Gln + Tyr104Ile
	Ser191Glu + Gly219Ser
	Asp 99Glu + Asn218Gln
35	Gly100Asn + Ser105Glu
	Gly166Pro + Pro210Asn
	Gln 59Asn + Thr164Ser
	Leu126His + Tyr214Ala
	Thr 66Pro + Lys213Asp
40	Trp106His + Gly211Ser
	Tyr167Leu + Ser204Glu
	Val 95Thr + Ala133Gly
	Ile107Ser + Gln206Glu
	Phe189Tyr + Lys213Asp
45	Gly 65Asn + Asn218Asp
	Tyr167Val + Lys213Glu
	Gly 97Gln + Ser132Glu
	Asp 99Glu + Gly102Pro
	Leu126Cys + Ala216Asp
	Leu126Cys + Gly127Ser
50	Ser191Asp + Ala216Asn

	Gly100Gln + Gly154Asp
	Asn 61Asp + Gly211Ser
	Ser161Asp + Phe189Leu
	Ile205Gln + Ala216Glu
5	Asn 62Gln + Tyr217Leu
	Ile107Met + Ser161Asp
	Leu126Ile + Tyr217Ser
	Ala 98His + Ser162Asp
	Asn 61Asp + Gly128Ser
10	Asn155Glu + Gly215Gln
	Asn155Gln + Ser204Asp
	Asn155Glu + Thr220Gln
	Lys213Asp + Tyr217His
	Gly127Pro + Ser204Glu
15	Ser204Asp + Tyr217Ala
	Glu156Asp + Val203Gly
	Gly127Glu + Ala133His
	Gly100Asn + Gly131Ser
	Gly211Gln + Lys213Asp
20	Ala187Asp + Phe189Leu
	Ala216Glu + Tyr217Cys
	Ser204Asp + Ala216Thr
	Gly131Ser + Thr158Asp
	Gly100Asn + Gln206Asn
25	Ser105Asp + Gly131Gln
	Ser204Asp + Tyr214Val
	Tyr214Met + Tyr217Ile
	Ser 63Glu + Thr164Asn
	Ile107Cys + Ala216Pro
30	Trp106Gly + Gln206Asp
	Gly102Asp + Thr164Pro
	Asp 99Glu + Ala216Gln
	Lys213Glu + Ala216Gln
	Ala133Ser + Pro210Glu
35	Asp 60Glu + Tyr104Asn
	Asn 62Gln + Ile107Cys
	Tyr167Ala + Gly211Asp
	Glu156Asp + Tyr217Ile
	Gly131Pro + Leu209Pro
40	Lys213Glu + Asn218Gln
	Gly160Ser + Val203Glu
	Asn155Ser + Tyr167Ala
	Asp 60Glu + Phe189Gly
	Thr164Gln + Gly219Ser
45	Ser162Asp + Gln206Asn
	Gly100Glu + Tyr104Asn
	Gly160Pro + Gln206Ser
	Thr 66Gly + Ala216Gly
	Tyr104Ile + Gly215Pro
50	Pro201Gln + Ala216Thr

	Gln103Glu + Ala133Asn
	Ser163Glu + Phe189His
	Gly127Ser + Tyr217Ser
5	Gln206Asn + Leu209His
	Pro210Glu + Ala216Gln
	Asn 62Ser + Gln206Asn
	Ser161Glu + Gly219Asn
	Val203Gly + Asn212Glu
10	Ala 98Glu + Leu126Met
	Val165Gln + Ser204Asp
	Gly154Ser + Ala216His
	Pro201Gly + Gly211Glu
	Ser161Asp + Gly219Gln
15	Asn155Glu + Thr220Asn
	Leu 96Glu + Ile107Leu
	Thr158Ser + Gly215Ser
	Ser 63Glu + Pro129Ser
	Val 95Asn + Ser163Glu
20	Gly102Asn + Leu126Glu
	Thr 66Gly + Ala216Pro
	Gly157Ser + Thr158Glu
	Ala 98Asp + Ala187Ser
	Asp 99Glu + Thr164Gln
25	Thr 66Ser + Ser105Glu
	Gln103Asp + Gly154Pro
	Thr 66Glu + Tyr217His
	Gly127Gln + Ser204Glu
	Phe189Ile + Tyr217Thr
30	Ala133Gln + Lys213Asp
	Ser130Asp + Tyr217Thr
	Leu126Ile + Asn212Ser
	Gly154Asn + Gln206Asp
	Thr 66Pro + Glu156Asp
35	Gln103Asn + Lys213Asp
	Phe189Met + Gln206Asp
	Leu126Asn + Gly154Gln
	Pro210Gly + Gly215Glu
	Leu126Val + Ala216Pro
40	Gln206Ser + Tyr217His
	Leu 96Asn + Lys213Asp
	Leu126Pro + Ala216Ser
	Val203His + Gly211Asp
	Tyr167Ala + Tyr217Asp
	Trp106Asn + Gln206Asn
45	Gly127Ser + Ser161Glu
	Lys213Glu + Gly219Asn
	Val 95Thr + Thr208Gly
	Thr158Gly + Ser204Glu
	Gly 97Pro + Trp106Tyr
50	Phe189Ile + Val203His

	Leu 96Gln + Lys213Glu
	Gln206Glu + Ala216Thr
	Gly154Ser + Asn155Glu
5	Ser132Asp + Tyr214Asn
	Pro129Gln + Ala133Pro
	Ala 98Asn + Gly127Asp
	Gly211Gln + Asn218Asp
	Trp106Cys + Ser163Asp
10	Leu 96His + Ala216Gly
	Gly 97Asn + Ser204Asp
	Asn 61Ser + Gly157Asp
	Pro210Asn + Tyr217His
	Asp 60Glu + Tyr104Ala
	Thr164Asn + Ala200Gly
15	Tyr214Val + Ala216Asp
	Leu126His + Ala216Ser
	Gly128Gln + Asn212Asp
	Ser162Glu + Gln206Ser
	Gln206Glu + Ala216Ser
20	Thr164Pro + Thr220Asp
	Val203Ser + Gly219Asp
	Gln206Asn + Gly219Asp
	Ser 63Asp + Ile107Gln
25	Gly102Gln + Val203Ala
	Ser101Glu + Val165Gln
	Gln 59Ser + Gly166Glu
	Ser101Glu + Tyr217Ser
	Gly131Asn + Ala187Glu
	Gly102Ser + Tyr214Gly
30	Thr158Ser + Thr220Glu
	Asp 99Glu + Gly215Gln
	Val 95Gly + Thr220Asp
	Ala200Ser + Tyr214Val
	Ser188Glu + Ala216Asn
35	Tyr214His + Ala216Asp
	Thr158Glu + Phe189Asn
	Asn155Gln + Ser191Asp
	Thr 66Ser + Leu126Ser
	Thr 66Gly + Gln206Asp
40	Ser105Asp + Tyr214Thr
	Gly102Pro + Thr164Gln
	Trp106Gly + Pro210Gly
	Asn155Asp + Thr220Gln

TABLE 23

## Multi-loop Triple Mutation Variants

Gln 59Ser + Leu 96Gly + Ser204Glu  
 Asn 62Ser + Val 95Gln + Asn218Asp  
 Tyr104Cys + Gly127Gln + Lys213Glu

5 Ser188Glu + Gly215Asn + Ala216Pro  
Gly 97Gln + Ile107Ala + Gly157Glu  
Ser162Glu + Pro210Gln + Gly215Asn  
Thr 66Pro + Val203Cys + Tyr217Ser  
Ser105Glu + Ala133Gly + Phe189Val  
Leu 96Asn + Asn212Ser + Tyr214Glu  
Gln 59Asp + Gly131Gln + Leu209Pro  
Trp106Pro + Gly157Glu + Tyr217Ile  
10 Thr 66Gln + Leu126Asn + Ser188Glu  
Asn212Ser + Lys213Asp + Gly219Gln  
Val 95Asp + Leu126Ser + Asn155Gln  
Asn 61Glu + Thr 66Pro + Phe189Pro  
Gly160Asp + Gly166Asn + Ala216Asn  
Trp106Asn + Gly127Ser + Val203Asn  
15 Ser101Asp + Ile107Ala + Gly202Ser  
Ala133Thr + Phe189Ser + Tyr214Ile  
Gln 59Asn + Gly 97Asp + Trp106Phe  
Gly157Pro + Pro210Gly + Ala216Glu  
Gly160Ser + Asn212Ser + Tyr217Thr  
20 Asn 62Gln + Gln206Asn + Ala216Ser  
Pro129Ser + Gly215Glu + Tyr217Pro  
Ala 98Asn + Tyr217His + Thr220Gly  
Val203Gly + Gly211Glu + Ala216Asn  
Gly127Glu + Tyr214Asn + Ala216His  
25 Trp106Pro + Ala133Pro + Gln206Asp  
Val 95Ser + Gly128Glu + Tyr217Cys  
Ser159Asp + Gly166Gln + Gly219Gln  
Leu 96Val + Glu156Asp + Gly157Pro  
Ala133Gly + Thr208Pro + Tyr214Pro  
30 Trp106Asn + Gly128Pro + Val203Met  
Gly 65Ser + Gly102Asn + Ala187His  
Ala200Gln + Gln206Glu + Tyr217His  
Gln103Ser + Glu156Asp + Ala216Ser  
Gln 59Asn + Ala216Thr + Gly219Pro  
35 Gly102Ser + Pro210Asp + Tyr217Ile  
Gly100Glu + Ile107Ser + Thr158Gly  
Ala 98Glu + Gly154Gln + Pro210Gln  
Gln103Glu + Leu126Pro + Ala216His  
Lys213Glu + Ala216His + Tyr217Leu  
40 Gly154Glu + Tyr167Thr + Tyr217Ser  
Gly 97Ser + Trp106Ile + Ala216Gly  
Gly102Ser + Phe189Gly + Gly219Asn  
Gly157Pro + Gly160Asp + Val203Cys  
Leu 96Met + Ala 98Gly + Gly100Ser  
45 Gly127Asn + Gly160Pro + Gln206Glu  
Leu 96Thr + Tyr217Ala + Gly219Asn  
Trp106Phe + Lys213Glu + Tyr217Thr  
Gly102Glu + Gly127Asn + Gly128Gln  
Ala133Asn + Gly154Asn + Ser161Asp  
50 Asn 61Gln + Gln206Ser + Ala216Asn

	Ser204Asp + Gly219Ser + Thr220Gly
	Ala 98Gln + Ser159Glu + Tyr217Asn
	Gly 97Asp + Gly100Pro + Phe189Gln
5	Gln206Ser + Gly211Asn + Tyr217His
	Ala 98Gly + Ala187Asn + Ser188Asp
	Asp 99Glu + Thr164Asn + Phe189Cys
	Trp106Cys + Gly157Ser + Gln206Ser
	Gly157Asn + Ser188Asp + Tyr217Gly
10	Gly166Ser + Ser188Asp + Ala216Asn
	Leu126Asn + Gly128Asn + Ala216Ser
	Leu 96His + Ser132Asp + Tyr217Ala
	Ala 98His + Lys213Glu + Tyr217Met
	Ala 98Pro + Ser130Asp + Gly160Asn
15	Ser130Asp + Ala133Gly + Thr220Gly
	Asn 61Gln + Ile107His + Asn218Glu
	Gln206Glu + Tyr217Cys + Thr220Gly
	Gly157Ser + Pro210Asp + Tyr214Gln
	Val 95Gln + Gly102Pro + Gly166Glu
20	Tyr104Ile + Ser191Glu + Gly219Ser
	Asp 99Glu + Gly100Asn + Asn218Gln
	Gly131Glu + Gly166Pro + Pro210Asn
	Leu126His + Thr164Ser + Tyr214Ala
	Thr 66Pro + Gly211Ser + Lys213Asp
25	Trp106His + Tyr167Leu + Ser204Glu
	Val 95Thr + Ala133Gly + Gln206Glu
	Gly 97Gln + Gly102Pro + Ser132Glu
	Leu126Cys + Ser191Asp + Ala216Asn
	Gly100Gln + Gly154Asp + Gly211Ser
30	Asn 62Gln + Ala216Glu + Tyr217Leu
	Leu126Ile + Ser161Asp + Tyr217Ser
	Pro129Glu + Asn155Gln + Thr158Gln
	Gly127Glu + Ala133His + Val203Gly
	Gly131Ser + Gly211Gln + Lys213Asp
	Gly131Ser + Thr158Asp + Ala216Thr
35	Gly100Asn + Ser105Asp + Gln206Asn
	Gly 97Glu + Gly160Gln + Thr164Asn
	Ile107Cys + Lys213Asp + Ala216Pro
	Trp106Gly + Gln206Asp + Ala216His
	Ala133Ser + Lys213Glu + Ala216Gln
40	Asn 62Gln + Ile107Cys + Thr164Asp
	Gly131Pro + Leu209Pro + Tyr217Ile
	Asn155Ser + Tyr167Ala + Phe189Gly
	Asp 60Glu + Thr164Gln + Gly219Ser
	Gly160Pro + Ser204Glu + Gln206Ser
45	Thr 66Gly + Gly100Asp + Ala216Gly
	Tyr104Ile + Gly215Pro + Ala216Thr
	Gly127Ser + Lys213Asp + Tyr217Ser
	Ser188Glu + Gln206Asn + Leu209His
	Asn 62Ser + Gln206Asn + Pro210Glu
50	Ala 98Glu + Leu126Met + Val203Gly

5 Gly154Ser + Ser161Glu + Ala216His  
Pro201Gly + Gly211Glu + Ala216Thr  
Ser161Asp + Gly219Gln + Thr220Asn  
Asn 62Glu + Thr158Ser + Gly215Ser  
Gly102Asn + Leu126Glu + Ala216Pro  
Gly127Gln + Ser204Glu + Tyr217Thr  
Ala133Gln + Phe189Ile + Lys213Asp  
Ser130Asp + Asn212Ser + Tyr217Thr  
10 Leu126Ile + Gly154Asn + Gln206Asp  
Thr 66Pro + Gln103Asn + Lys213Asp  
Leu126Asn + Gly154Gln + Pro210Gly  
Leu126Val + Gly215Glu + Ala216Pro  
Gln206Ser + Lys213Asp + Tyr217His  
Leu 96Asn + Leu126Pro + Ala216Ser  
15 Ser 63Asp + Trp106Asn + Gln206Asn  
Gly127Ser + Ser161Glu + Gly219Asn  
Val 95Thr + Thr208Gly + Lys213Glu  
Gly 97Pro + Trp106Tyr + Asn218Glu  
Leu 96Gln + Phe189Ile + Val203His  
20 Ser132Asp + Ala133Pro + Tyr214Asn  
Ala 98Asn + Gly127Asp + Gly211Gln  
Leu 96His + Gly 97Asn + Ala216Gly  
Pro210Asn + Gly215Glu + Tyr217His  
Asp 60Glu + Trp106Tyr + Pro129Gln  
25 Gly157Asn + Phe189Val + Asn218Asp  
Gly100Asp + Thr164Asn + Ala200Gly  
Leu126His + Gln206Asp + Ala216Ser  
Ser 63Asp + Ile107Gln + Val203Ala  
Ser101Glu + Gly102Gln + Val165Gln  
30 Asp 99Glu + Thr158Ser + Gly215Gln  
Ala200Ser + Ser204Glu + Tyr214Val  
Asn155Gln + Thr158Glu + Phe189Asn  
Thr 66Gly + Ser105Asp + Tyr214Thr  
Gly102Pro + Thr164Gln + Pro210Gly  
35 Trp106Gly + Asn155Asp + Thr220Gln  
Thr158Gly + Ala187Gln + Ser204Glu  
Gly154Gln + Tyr167Cys + Ser204Glu  
Asp 60Glu + Ala 98His + Gly102Pro  
Gly131Ser + Ile205Val + Ala216Asp  
40 Gly128Gln + Val165Cys + Gly211Gln  
Gly 97Asn + Ile107Gln + Gly166Gln  
Gly160Asp + Gly166Pro + Tyr214Ile  
Gln 59Asp + Gly154Ser + Asn218Gln  
Gly154Ser + Val165His + Ser204Glu  
45 Ser 63Glu + Pro129Ser + Tyr217Gly  
Gly157Pro + Thr158Ser + Lys213Glu  
Thr164Glu + Gly215Ser + Ala216Asn  
Thr 66Pro + Asp 99Glu + Tyr217Cys  
Trp106Met + Ala187Ser + Tyr217Ile  
50 Ile107Thr + Glu156Asp + Tyr217Cys

5 Leu126Pro + Gly131Asn + Tyr217Leu  
Tyr167His + Gly219Pro + Thr220Glu  
Val 95Pro + Trp106Ile + Tyr217Gly  
Val 95His + Gln206Asn + Lys213Glu  
Val 95Ala + Ala187Ser + Tyr217Glu  
Asp 60Glu + Asn 62Gln + Tyr167Ile  
Gly160Asn + Ala187Gly + Gln206Ser  
Gly102Gln + Trp106His + Ser163Glu  
10 Asn 62Gln + Ser188Glu + Pro210Gln  
Gly100Pro + Gly202Gln + Ala216Ser  
Ser105Glu + Ile107Thr + Gly131Pro  
Thr 66Gly + Gly131Asp + Phe189Ser  
Gln103Asn + Ala187Ser + Ser204Glu  
Asp 60Glu + Thr164Pro + Ala216Ser  
15 Gln 59Glu + Asn212Ser + Tyr217Ser  
Asn 61Glu + Gly166Gln + Gly215Pro  
Asn 62Gln + Gly160Gln + Gly219Ser  
Ser105Glu + Tyr167Ala + Tyr217Ser  
Gly100Ser + Asn155Ser + Tyr217Asn  
20 Gly 97Pro + Leu126Ala + Gly157Gln  
Gly100Ser + Gly131Gln + Phe189Glu  
Ser132Asp + Ala187Pro + Gln206Asn  
Gln 59Asp + Gln206Asn + Tyr217Ile  
Gln103Asn + Ile107Asn + Ala133Ser  
25 Gly128Gln + Pro129Asn + Ala216Asp  
Thr 66Glu + Trp106Ala + Ala187Ser  
Asp 60Glu + Gly 65Asn + Tyr214Ser  
Ser132Asp + Gly157Asn + Ala216Ser  
Asn 62Asp + Ile205Thr + Gln206Ser  
30 Gln 59Asn + Gly 65Pro + Val 95Asp  
Val 95Ser + Gly102Ser + Lys213Asp  
Ala216Pro + Tyr217Pro + Asn218Ser  
Ser 63Asp + Gly127Ser + Thr220Asn  
Gly 97Asn + Gly154Gln + Ala216Asn  
35 Ala 98His + Trp106Val + Ala216Gln  
Gly102Asn + Ile107Gln + Ser162Asp  
Ile107Val + Lys213Glu + Ala216Ser  
Tyr104Leu + Gln206Glu + Thr220Asn  
Pro201Asn + Pro210Asn + Gly211Gln  
40 Gly166Asn + Ile205Asn + Ala216Thr  
Ala 98Ser + Gln206Ser + Gly215Ser  
Ala133His + Ser188Asp + Tyr217Gly  
Ala 98Glu + Gly131Pro + Gly157Pro  
Leu 96Ile + Ser188Asp + Val203His  
45 Tyr167Thr + Gln206Ser + Tyr217His  
Leu 96Gln + Ser161Glu + Ala216Thr  
Gly127Glu + Thr158Pro + Pro201Gly  
Gly160Ser + Lys213Glu + Ala216Ser  
Tyr104Ser + Leu126His + Tyr214His  
50 Asn 62Ser + Gly160Glu + Ala216His



5 Leu 96Cys + Thr164Ser + Ser204Asp  
Gly131Gln + Phe189Ile + Val203Asp  
Asp 60Glu + Gly 65Gln + Thr 66Asn  
Gly102Glu + Gly128Ser + Ala216Gln  
Asn 62Gln + Val 95Gly + Gln206Asn  
Gly 97Pro + Gly154Asp + Asn218Gln  
Thr 66Pro + Leu 96Val + Ala216Pro  
Gly 97Asn + Asn155Glu + Tyr214Val  
10 Tyr104Ala + Tyr167Glu + Ala216Pro  
Gly157Asn + Asn218Glu + Thr220Gly  
Ala133His + Thr164Gln + Gly166Ser  
Leu126Gln + Ser159Glu + Gly160Asp  
Asn 61Asp + Asn 62Asp + Gly128Ser  
Thr 66Pro + Gly100Glu + Ser101Glu  
15 Ser204Glu + Ile205Gln + Ala216Glu  
Ser204Asp + Ala216Glu + Tyr217Cys  
Ser204Asp + Ala216Asp + Thr220Gln  
Gln103Asn + Ser204Glu + Ala216Glu  
Gly202Gln + Ser204Glu + Asn218Asp  
20 Ser204Glu + Gln206Asp + Ala216Asp  
Ser204Asp + Gln206Glu + Ala216Asp  
Tyr167Ala + Ser204Asp + Tyr217Asp  
Gly211Asp + Lys213Glu + Ala216Thr  
Gly211Asp + Lys213Glu + Tyr217Pro  
25 Tyr167Val + Gly211Asp + Lys213Glu  
Asp 60Glu + Asn 62Asp + Tyr217Leu  
Gly160Glu + Ser162Glu + Ala216Thr  
Ser204Glu + Gln206Asp + Tyr217Leu  
Ser204Glu + Gln206Glu + Ala216Thr  
30 Ile107Cys + Ser204Glu + Gln206Glu  
Ser204Glu + Gln206Glu + Gly215Asn  
Ser161Asp + Ser163Asp + Ala216His  
Thr164Pro + Gln206Glu + Tyr217Asp  
Asp 60Glu + Gln206Asn + Pro210Asp  
35 Asp 60Glu + Tyr104Asn + Pro210Glu  
Ala187Glu + Val203Glu + Asn218Glu  
Ser130Glu + Gly166Glu + Phe189Tyr  
Thr158Asp + Ser162Glu + Gln206Ser  
Gly154Asp + Val203Ser + Gly219Asp  
40 Ser188Glu + Ser191Asp + Ala216Asn  
Asp 60Glu + Gly 97Glu + Asp 99Glu  
Thr164Pro + Ser204Glu + Gly219Glu  
Asp 99Glu + Gly102Asp + Ala216Gln  
Ser204Glu + Gln206Asn + Gly215Asp  
45 Ser204Asp + Gln206Asp + Tyr214Asp  
Thr 66Asp + Gly211Glu + Lys213Asp  
Ser101Glu + Leu126Glu + Tyr214His  
Asn 61Glu + Leu 96Glu + Ile107Leu  
Asp 60Glu + Leu 96Glu + Gly166Pro  
50 Ser101Glu + Gly127Glu + Ala187Gln

5 Ser 63Glu + Gly131Asn + Lys213Glu  
Ser 63Asp + Phe189Leu + Lys213Glu  
Ser105Glu + Ser132Glu + Tyr167Gly  
Ser204Asp + Ala216Glu + Thr220Glu  
Ser204Glu + Lys213Asp + Gly215Asp  
Asp 99Glu + Ser101Asp + Tyr104Asp  
Ser 63Asp + Pro210Glu + Tyr217Glu  
Thr158Gln + Gln206Asp + Lys213Asp  
10 Gln206Glu + Lys213Glu + Ala216His  
Gly157Asp + Tyr214Gly + Thr220Asp  
Ser 63Glu + Gly100Ser + Tyr217Asp  
Gly100Glu + Gln103Asp + Gln206Asn  
Gly154Glu + Ser163Asp + Val203Met  
Val 95Gly + Lys213Asp + Ala216Glu  
15 Gln 59Asn + Leu126Glu + Pro129Glu  
Ser204Glu + Gln206Asp + Lys213Glu  
Ala187Asp + Ser204Glu + Gln206Glu  
Ser 63Glu + Ser204Glu + Ala216Asp  
Asn 61Asp + Ser 63Asp + Ala216Glu  
20 Pro129Glu + Asn155Glu + Ser163Asp  
Ser 63Asp + Ile107Leu + Asn212Asp  
Gln206Asp + Pro210Asp + Asn212Asp  
Glu156Asp + Ser163Glu + Gly219Asp  
Ile107Glu + Gly131Ser + Ser132Asp  
25 Gly100Asn + Gly211Asp + Gly215Glu  
Gln103Asp + Gly127Glu + Ala216Gln  
Ser130Asp + Gly131Asp + Lys213Glu  
Gly100Asp + Ser101Glu + Ser163Asp  
Pro129Asp + Ser130Asp + Tyr217Glu  
30 Val203Asp + Ser204Glu + Lys213Glu  
Ser132Asp + Ala216Glu + Tyr217Glu  
Ser101Glu + Ala187Glu + Ser188Glu  
Ala 98Asp + Asp 99Glu + Ser204Asp  
Ser204Asp + Gln206Asp + Asn212Asp  
35 Gln103Asp + Glu156Asp + Ser191Glu  
Ser132Asp + Ser204Glu + Ala216Asp  
Ala 98Glu + Ser204Glu + Ala216Glu  
Ser204Asp + Lys213Asp + Asn218Glu  
Ser204Glu + Gly211Asp + Tyr217Asp  
40 Ser162Asp + Gly166Asp + Asn212Ser  
Gly128Glu + Gly166Glu + Gln206Glu  
Asp 60Glu + Asn 62Glu + Ser204Asp  
Asp 99Glu + Ser101Asp + Gly154Glu  
Gln103Ser + Gln206Glu + Gly219Asp  
45 Phe189Asp + Pro210Asp + Lys213Glu  
Asn 61Asp + Ser101Glu + Gly128Asp  
Thr 66Glu + Gly166Gln + Ala216Glu  
Ser101Glu + Ser204Glu + Gln206Asp  
Gly157Glu + Ser204Glu + Gln206Glu  
50 Asp 99Glu + Ser204Asp + Gln206Glu

5 Gly 97Glu + Ser204Glu + Gln206Asp  
Ser101Asp + Gly102Ser + Ser105Asp  
Ser161Glu + Ser163Asp + Gln206Asp  
Ser130Asp + Ser132Glu + Asn212Glu  
Ser130Glu + Ser132Glu + Gly160Asp  
Pro129Glu + Gly131Glu + Gly215Glu  
Asn 62Gln + Thr158Asp + Gly166Glu  
Ser132Glu + Gln206Glu + Tyr217Asp  
10 Asp 60Glu + Phe189His + Asn212Glu  
Gly131Glu + Lys213Asp + Gly215Glu  
Ser159Glu + Ser163Glu + Ser204Glu  
Thr158Glu + Ser162Asp + Gly219Asp  
Tyr104Glu + Ser132Glu + Asn212Asp  
Asp 99Glu + Glu156Asp + Ser159Glu  
15 Ser 63Glu + Ser188Asp + Ser191Asp  
Ser188Asp + Ser191Glu + Ala216Asp  
Gln 59Glu + Ser188Asp + Ser191Asp  
Ser204Glu + Lys213Glu + Gly219Glu  
Asp 60Glu + Ser204Asp + Gly219Asp  
20 Leu126Asp + Gly166Asp + Ser204Asp  
Thr164Glu + Ser188Glu + Gln206Ser  
Asp 60Glu + Gln206Glu + Lys213Asp  
Ser105Asp + Leu126Glu + Thr220Asp  
Asp 99Glu + Glu156Asp + Ser188Asp  
25 Gln 59Glu + Asn 62Asp + Ala187Glu  
Gly166Glu + Val203Asp + Gln206Glu  
Asn155Glu + Ala187Glu + Lys213Asp  
Thr 66Asp + Ser204Glu + Lys213Asp  
Ser 63Asp + Ser188Glu + Asn218Glu  
30 Ser 63Asp + Ser105Asp + Lys213Asp  
Ser105Asp + Ser132Glu + Gln206Glu  
Ser 63Asp + Gly 97Asp + Asn155Asp  
Ser 63Glu + Ser101Asp + Ser105Asp  
Thr164Glu + Gln206Glu + Lys213Glu  
35 Leu126Glu + Gln206Asp + Lys213Asp  
Gly131Glu + Gln206Asp + Lys213Asp  
Ser 63Asp + Trp106Asp + Tyr217Glu  
Gly160Glu + Lys213Glu + Ala216Glu  
Ala133Glu + Lys213Asp + Ala216Asp  
40 Ser 63Glu + Gln206Asp + Gly215Gln  
Lys213Asp + Ala216Asn + Tyr217Glu  
Ser130Asp + Ala187Asp + Ser204Glu  
Asp 99Glu + Ser188Glu + Asn218Asp  
Asn 61Asp + Ser188Glu + Asn218Glu  
45 Gly102Asp + Ser204Glu + Thr220Glu  
Gly127Asp + Ser191Glu + Lys213Asp  
Thr 66Glu + Gly 97Glu + Tyr217Cys  
Gly154Asp + Ala187Glu + Gly215Asp  
Gly102Asp + Gly154Glu + Ser188Glu  
50 Gln103Asp + Ser132Asp + Gln206Glu

Tyr167His + Ser191Glu + Asn218Asp  
 Asp 60Glu + Glu156Asp + Gly160Glu  
 Gln103Glu + Gly154Glu + Asn218Asp  
 Asp 60Glu + Asn155Glu + Ser159Asp  
 5 Gln103Glu + Ser161Glu + Ser191Asp  
 Ala 98Asp + Ser132Asp + Gly166Glu  
 Ser188Asp + Ser204Asp + Tyr214Val

TABLE 24

	Multi-loop Quadruple Mutation Variants
10	Gln 59Ser + Asn 62Ser + Leu 96Gly + Ser204Glu
	Gly127Gln + Ser188Glu + Gly215Asn + Ala216Pro
	Asn 62Gln + Ile107Ala + Gln206Asp + Tyr217Thr
	Asn 61Ser + Leu 96His + Gly157Pro + Ala216Gly
15	Leu 96Gln + Gly127Gln + Glu156Asp + Thr220Asn
	Thr158Glu + Gly202Ser + Gln206Ser + Thr220Ser
	Gly 97Asn + Ser105Asp + Gly215Ser + Ala216Ser
	Leu126Thr + Gly211Gln + Lys213Asp + Ala216Ser
	Gly100Asp + Trp106Asn + Gly127Ser + Val203Asn
20	Ile107Ala + Gly160Asn + Gly166Asp + Gly202Ser
	Ala133Thr + Phe189Ser + Tyr214Ile + Ala216Glu
	Asn 62Ser + Ser163Asp + Phe189Ser + Pro201Gln
	Ala 98Gln + Gly102Asn + Pro201Asn + Gly219Asp
	Thr 66Ser + Leu126Glu + Gly127Gln + Ala216Thr
25	Pro129Ser + Thr164Gln + Ala216Asp + Tyr217Val
	Gly128Gln + Thr158Gln + Gln206Asn + Asn212Asp
	Gly157Ser + Gln206Glu + Tyr217Cys + Thr220Gly
	Val 95Gln + Tyr104Ile + Ser191Glu + Gly219Ser
	Gln 59Asn + Gly 97Asn + Gly154Pro + Asn218Ser
30	Pro129Gly + Thr158Asn + Gln206Asn + Gly211Pro
	Ala 98His + Trp106His + Gln206Asn + Lys213Asp
	Leu126Ile + Ser204Glu + Gln206Asn + Tyr217Thr
	Gln 59Glu + Asn 62Gln + Phe189Leu + Val203Ala
	Pro129Gln + Gly154Pro + Ala187Thr + Lys213Glu
35	Ser 63Glu + Thr164Asn + Gln206Ser + Pro210Asn
	Leu 96Met + Gln103Asn + Ala133Ser + Ser204Glu
	Trp106Ala + Gly154Pro + Ala187Asn + Gly219Pro
	Asn 62Glu + Gly102Pro + Gly160Asn + Asn218Ser
	Thr 66Gly + Gly100Asp + Tyr104Ile + Ala216Gly
40	Gly102Asp + Pro201Gln + Gly215Pro + Ala216Thr
	Leu126Met + Val203Gly + Asn212Glu + Gly219Asn
	Leu 96Glu + Ile107Leu + Thr158Ser + Gly215Ser
	Ser130Asp + Ala133Gln + Asn212Ser + Tyr217Thr
	Thr 66Gly + Gly100Ser + Leu126Gly + Ala216Glu
45	Gln103Asp + Tyr104Ile + Gly128Gln + Tyr217Cys
	Leu126Pro + Ser204Asp + Gln206Asn + Thr208Asn
	Pro129Ser + Gly157Asn + Thr164Glu + Ala200Ser
	Gly128Gln + Val165Cys + Gly211Gln + Lys213Glu
	Gly160Asp + Gly166Pro + Gly211Ser + Tyr214Ile

5 Gln103Ser + Gly166Asn + Tyr214Ile + Gly215Pro  
 Asn 61Asp + Tyr104Ser + Leu126His + Tyr214His  
 Gly 65Gln + Gly131Gln + Phe189Ile + Val203Asp  
 Asn 62Gln + Thr 66Asp + Val 95Gly + Gln206Asn  
 Thr 66Pro + Gly 97Pro + Gly154Asp + Ala216Pro  
 Val 95Pro + Tyr104Gly + Gly127Ser + Gly215Asp  
 Asp 99Glu + Trp106Ala + Pro201Gln + Ala216Gly  
 Asn 61Gln + Val 95Asp + Gly102Asn + Ala187Asn  
 10 Ile107Gln + Val203Ser + Ser204Asp + Gly215Ser  
 Val 95Thr + Gly202Gln + Ser204Asp + Ala216Asn  
 Thr158Pro + Val203Gly + Lys213Glu + Tyr217Ser  
 Trp106Pro + Asn155Asp + Gln206Ser + Tyr214Ala  
 Gly102Asn + Gly157Ser + Tyr167Ala + Ala216Asn  
 Gly160Asn + Val203Thr + Pro210Glu + Asn218Gln  
 15 Ile107Ser + Gly128Asn + Asn155Glu + Ala216Gly  
 Gln103Asn + Pro129Gly + Gly166Gln + Thr220Gly  
 Asn 61Ser + Ser 63Asp + Thr 66Gly + Gly154Ser  
 Tyr104Gly + Pro129Ser + Gln206Ser + Gly219Ser  
 Gly102Pro + Gly131Asp + Asn155Ser + Tyr217His  
 20 Asn 61Ser + Val 95Gln + Ser204Asp + Ala216Gln  
 Thr158Asn + Ala187Gly + Tyr217Ala + Gly219Asp  
 Gly 65Gln + Gly 97Pro + Ser130Glu + Pro210Asn  
 Gly128Asn + Ser159Glu + Pro201Ser + Tyr217Val  
 Leu126Asn + Asn155Gln + Gly202Gln + Asn212Ser  
 25 Thr 66Ser + Tyr104Val + Gly154Glu + Gly215Asn  
 Gly102Asn + Gly128Gln + Ser161Glu + Tyr217Met  
 Ser132Glu + Thr158Gln + Thr164Asn + Gln206Asn  
 Asn 62Glu + Leu 96Ile + Gly211Ser + Gly219Ser  
 Thr208Pro + Pro210Gly + Ala216Thr + Tyr217Met  
 30 Gly100Gln + Gly160Asn + Pro201Gly + Asn212Asp  
 Tyr104Asp + Gly154Pro + Ala187Asn + Val203Ser  
 Leu 96Gln + Leu126Thr + Ser162Glu + Tyr217Val  
 Gly128Asn + Ala187Pro + Pro201Gly + Ser204Glu  
 Gln103Ser + Gly157Glu + Thr158Gln + Ala216Gln  
 35 Leu126Ser + Thr164Glu + Val203Pro + Gly211Gln  
 Thr164Gly + Val203Met + Ala216Asp + Tyr217Gln  
 Ser159Asp + Val203Asn + Ile205Asn + Pro210Ser  
 Gly 65Asn + Gln206Asp + Ala216Gly + Tyr217His  
 Gln103Asn + Ile107Cys + Thr164Asp + Val203Thr  
 40 Gly128Glu + Asn155Gln + Thr158Ser + Gly160Ser  
 Ala 98His + Ser162Glu + Gln206Asn + Tyr217Gly  
 Gly128Ser + Thr164Asn + Ser204Glu + Tyr217Gly  
 Gly127Gln + Gly157Ser + Ser159Asp + Tyr217Val  
 Gly157Asn + Gln206Asn + Tyr217Val + Gly219Pro  
 45 Thr 66Ser + Ala133Thr + Ser163Asp + Thr208Gln  
 Leu 96Thr + Gly131Asp + Gln206Asn + Ala216Gly  
 Asn 61Ser + Ser132Glu + Gly211Ser + Asn218Gln  
 Gly100Ser + Tyr104Ala + Ser204Asp + Gly211Gln  
 Leu 96His + Ala 98Glu + Pro129Gln + Ala133Asn  
 50 Asn 62Glu + Gly128Gln + Ala187Asn + Gly215Ser

Leu 96Ile + Gly157Ser + Val203Ala + Ala216Ser  
Asn 61Gln + Val 95Thr + Gly160Asp + Ala216His  
Leu 96Cys + Gly128Pro + Ser191Glu + Thr208Asn  
Trp106Ala + Gly131Gln + Val203Ala + Tyr214Gln  
5 Asn 61Ser + Ala216Gln + Tyr217Leu + Gly219Asn  
Tyr104Gly + Ser105Glu + Thr158Ser + Leu209Thr  
Ala133Ser + Phe189Thr + Asn212Glu + Tyr217Thr  
Tyr104Ser + Thr158Gly + Thr164Glu + Ala216Pro  
Gln 59Asn + Thr 66Asn + Thr164Gly + Ala187Pro  
10 Ile107His + Gly157Ser + Lys213Glu + Tyr217Asn  
Gly127Ser + Gln206Asp + Gly215Gln + Tyr217Leu  
Leu126Gly + Gly131Glu + Tyr167Met + Thr220Gln  
Thr158Gln + Lys213Glu + Gly215Ser + Tyr217Gly  
Asn 61Gln + Leu126Gly + Thr164Ser + Asn218Asp  
15 Asn 62Asp + Pro129Gly + Gln206Ser + Ala216His  
Asp 60Glu + Val 95Gln + Leu126Pro + Val203Thr  
Gln103Glu + Ile107Val + Phe189Asn + Ala216Thr  
Ile107Thr + Pro129Gln + Lys213Glu + Tyr217Thr  
Tyr104His + Gly154Gln + Gly157Asp + Tyr217Ser  
20 Gln 59Asn + Trp106Cys + Ala200Thr + Ala216Gln  
Thr 66Gln + Gly 97Ser + Gly127Pro + Tyr217Asp  
Gly100Asn + Ser204Asp + Pro210Ser + Tyr214Gly  
Asn 62Ser + Ile107Gly + Leu126Cys + Thr220Gly  
Leu126His + Gly154Asp + Asn218Gln + Thr220Asn  
25 Ser101Glu + Gly157Gln + Tyr214Pro + Ala216His  
Asn 62Gln + Ser162Glu + Val203Ser + Ala216Thr  
Tyr104Gln + Trp106Gly + Leu126Asp + Asn212Gln  
Gln 59Ser + Val 95Pro + Gly202Asn + Tyr217Ser  
Leu 96Pro + Gly160Asp + Ser161Glu + Gly166Asn  
30 Ser159Glu + Gly160Asp + Tyr167Gly + Phe189Val  
Asn212Glu + Lys213Glu + Ala216Ser + Tyr217Gln  
Thr158Asp + Ser159Asp + Gly215Asn + Ala216Thr  
Ala 98Asp + Asp 99Glu + Thr164Gln + Ala187Ser  
Gly 97Pro + Gly131Pro + Gly154Asp + Asn155Asp  
35 Gly102Ser + Trp106Gln + Gly157Glu + Phe189Asp  
Gly100Gln + Ser204Glu + Tyr214Ile + Ala216Glu  
Val 95Pro + Ser204Glu + Ala216Gly + Asn218Glu  
Ser204Glu + Ile205Gln + Pro210Gly + Asn218Asp  
Gly 97Ser + Gly154Asn + Gln206Asp + Gly215Asp  
40 Gly 97Asp + Ala 98Gln + Asp 99Glu + Gly154Ser  
Thr158Gln + Val165Met + Gly211Glu + Lys213Glu  
Gly160Glu + Ser162Asp + Tyr167Ile + Gly219Ser  
Asn 61Ser + Thr 66Ser + Asn155Glu + Gly157Asp  
Thr158Asp + Ser159Asp + Thr164Asp + Gly211Asn  
45 Val 95Asp + Gly102Glu + Ala187Pro + Tyr217Pro  
Asn 62Glu + Gly100Asp + Thr208Asn + Tyr217His  
Ser204Asp + Gln206Glu + Gly211Gln + Ala216His  
Gly154Asn + Ser204Glu + Gln206Asp + Tyr217Thr  
Thr 66Gln + Ser130Glu + Ser132Asp + Thr158Pro  
50 Asp 60Glu + Gly 65Asn + Thr 66Glu + Tyr214Ser

5 Asp 60Glu + Gln206Ser + Pro210Glu + Gly219Ser  
Thr158Asp + Ser163Glu + Ser191Glu + Ile205Gly  
Ser204Asp + Gly215Gln + Ala216Glu + Gly219Asp  
Thr158Asp + Ala187Asp + Phe189Glu + Tyr217Met  
Gly128Gln + Pro129Asn + Val203Asp + Ala216Asp  
Gly 97Asn + Ile107Gln + Ser204Glu + Gly219Glu  
Trp106Asn + Gly157Gln + Ser204Asp + Gly219Asp  
Gly127Asp + Gly128Asn + Ser130Asp + Gly219Gln  
Val 95Ser + Pro129Gly + Asn155Glu + Ser188Glu  
10 Asn155Asp + Ser188Asp + Phe189Asn + Ala216Gly  
Trp106Phe + Ser204Asp + Gln206Asp + Tyr214Asp  
Asn 62Asp + Gly 97Gln + Pro210Asp + Gly211Glu  
Val 95Asp + Tyr104Asp + Leu126Ser + Asn155Gln  
Gly100Asn + Gln206Asp + Lys213Glu + Ala216Asp  
15 Gln206Asp + Lys213Asp + Ala216Glu + Tyr217Asn  
Gly102Gln + Asn155Glu + Val203Glu + Asn218Asp  
Gln 59Glu + Thr 66Glu + Gly102Pro + Gly166Gln  
Leu126Cys + Gly157Asp + Ser163Asp + Ala216His  
Thr 66Asp + Gln206Asp + Ala216Asp + Gly219Pro  
20 Asn 62Asp + Ser 63Glu + Gly131Asn + Lys213Glu  
Leu126Asn + Pro129Asn + Ser191Asp + Gly219Glu  
Thr 66Asp + Gly100Asn + Gly127Ser + Lys213Glu  
Ile107Val + Phe189Asp + Val203Glu + Ala216Gln  
Ser 63Asp + Val 95Ser + Lys213Asp + Ala216Ser  
25 Ile107His + Val203Cys + Tyr214Glu + Tyr217Asp  
Asn 62Ser + Ser105Asp + Trp106Gly + Ser132Asp  
Ser 63Glu + Leu 96Cys + Pro210Glu + Ala216Glu  
Ala187Gly + Gly215Asp + Tyr217Thr + Asn218Glu  
Gly160Ser + Gln206Glu + Lys213Glu + Ala216Ser  
30 Gly131Pro + Phe189Leu + Gln206Glu + Lys213Glu  
Pro129Asn + Ala133Gln + Gln206Glu + Lys213Glu  
Ala 98His + Gly154Glu + Ser163Asp + Tyr217Met  
Val203His + Gln206Glu + Gly211Glu + Lys213Asp  
Leu126Ala + Ser204Glu + Gln206Asp + Lys213Glu  
35 Ile107Leu + Gly157Glu + Val203His + Gly219Glu  
Ala 98Glu + Gly102Asp + Ser105Glu + Leu209Thr  
Thr 66Gln + Lys213Glu + Ala216Glu + Asn218Glu  
Ser204Glu + Gln206Asn + Pro210Glu + Gly215Asp  
Gly127Asp + Ser132Asp + Gly154Asp + Val165Gln  
40 Ser 63Glu + Val203His + Asn212Glu + Tyr217Leu  
Gln206Glu + Lys213Glu + Tyr217Ala + Asn218Glu  
Gln206Asp + Lys213Glu + Ala216Asn + Asn218Asp  
Gly157Pro + Ser188Glu + Ser204Glu + Ala216Asp  
Gln 59Glu + Thr 66Asp + Gly100Gln + Gly215Glu  
45 Trp106Ser + Ala187Asp + Gln206Glu + Tyr217Asp  
Ser159Glu + Asn212Gln + Gly215Asp + Ala216Glu  
Gly160Asp + Ser161Asp + Gln206Asp + Tyr214Asn  
Thr 66Glu + Tyr167Gln + Gln206Glu + Gly211Pro  
Pro129Asn + Ser163Glu + Tyr217Glu + Asn218Glu  
50 Asn155Glu + Glu156Asp + Ser204Glu + Tyr214Thr

Gln 59Asp + Ser162Asp + Ser163Glu + Ala216Thr  
 Leu126Pro + Ser162Glu + Ser163Glu + Tyr217Glu  
 Gly100Glu + Val203Cys + Asn212Asp + Lys213Glu  
 Ser105Glu + Ala187Ser + Val203Glu + Ser204Asp  
 5 Gln103Asp + Ser163Glu + Thr164Glu + Pro201Gln  
 Val 95Gln + Glu156Asp + Gly157Asp + Lys213Glu  
 Ser162Glu + Thr164Gln + Ala216Asp + Tyr217Glu  
 Asp 99Glu + Gly100Glu + Ser159Glu + Ala216Thr  
 Ala 98Glu + Asp 99Glu + Trp106Gly + Gly154Asp  
 10 Asn 62Glu + Ser 63Glu + Pro129Ser + Asn155Asp  
 Asn 61Glu + Gln206Glu + Ala216Glu + Tyr217Cys  
 Thr 66Pro + Gln103Asp + Glu156Asp + Ser191Asp  
 Asp 60Glu + Ser204Asp + Ala216Asp + Tyr217Ile  
 Ser105Asp + Ser204Asp + Gln206Ser + Ala216Glu  
 15 Thr158Asn + Ser162Asp + Ser204Asp + Asn218Asp  
 Gln 59Asp + Gly157Ser + Ser204Asp + Asn218Asp  
 Gly 97Ser + Gly128Glu + Gln206Glu + Gly215Asp  
 Trp106Asp + Val203Cys + Ser204Glu + Tyr217Glu  
 Ser105Glu + Ala187Thr + Ser204Glu + Tyr217Glu  
 20 Gly 97Asn + Asn155Glu + Ser163Glu + Tyr214Val  
 Val 95Asp + Trp106Glu + Ala187Pro + Val203Asp  
 Gln103Asp + Trp106Glu + Gly128Asn + Ser162Asp  
 Gly128Glu + Ser130Asp + Ser188Glu + Ala216Gln  
 Gln103Asp + Ser105Glu + Gly154Glu + Ala216Thr  
 25 Ser159Glu + Gly211Glu + Lys213Asp + Tyr217Gly  
 Gln 59Asn + Ser188Asp + Gly211Glu + Lys213Glu  
 Ile107Glu + Gly211Glu + Lys213Asp + Tyr217Gln  
 Ser159Asp + Ser162Glu + Pro210Glu + Ala216Asn  
 Asp 60Glu + Asn 62Asp + Ser191Asp + Tyr217Leu  
 30 Asp 60Glu + Ser 63Asp + Ile107Asn + Phe189Glu  
 Leu 96Cys + Gly166Asp + Pro210Asp + Lys213Asp  
 Val 95Glu + Ala 98Asn + Gly102Glu + Ser162Glu  
 Ser 63Asp + Tyr167His + Ala216Glu + Gly219Glu  
 Tyr104Asp + Thr158Asp + Ser191Glu + Asn218Ser  
 35 Gly154Pro + Ser159Glu + Ser204Asp + Gln206Asp  
 Gly102Glu + Ser204Asp + Gln206Glu + Tyr217His  
 Asn155Gln + Ser163Asp + Ser204Glu + Gln206Glu  
 Gly131Asp + Thr158Gln + Ser204Asp + Gln206Asp  
 Tyr167Asp + Ser204Glu + Gln206Glu + Tyr217Asn  
 40 Gly 97Asp + Ala133Gly + Ser204Asp + Gln206Asp  
 Gly127Asp + Ser204Glu + Gln206Glu + Tyr214Asn  
 Gly102Glu + Gly127Gln + Asn155Asp + Thr220Asp  
 Gly 97Glu + Ser130Glu + Tyr167Asp + Tyr217Val  
 Asn 62Glu + Ala187Gly + Pro210Asp + Ala216Glu  
 45 Ser101Asp + Ser105Asp + Ala216His + Tyr217His  
 Ser130Asp + Ser132Glu + Asn212Glu + Ala216Gln  
 Ser130Glu + Ser132Glu + Gly160Asp + Thr220Gly  
 Gly100Glu + Tyr104Thr + Ser130Asp + Ser132Asp  
 Gln 59Ser + Gly160Asp + Gln206Glu + Tyr217Asp  
 50 Gly127Asp + Pro129Glu + Ser188Asp + Gln206Asn



Ser159Asp + Thr164Glu + Phe189His + Lys213Glu  
Asn 61Asp + Gly 97Asp + Ser159Glu + Thr220Ser  
Ser159Glu + Ser163Glu + Ser204Glu + Tyr217Ser  
Thr158Asp + Ser162Glu + Ala187Pro + Ala216Glu  
5 Leu 96Val + Thr158Glu + Ser162Asp + Gly219Asp  
Asp 99Glu + Thr158Asp + Ser162Asp + Val203Met  
Val 95Asp + Gly131Asn + Ser163Asp + Ser191Glu  
Asn 61Glu + Asp 99Glu + Ser204Asp + Tyr217Gly  
Asn 62Asp + Gly166Ser + Ser204Asp + Gly215Glu  
10 Gly102Asp + Ser105Asp + Tyr167Ala + Gly211Glu  
Ser188Asp + Ser191Glu + Ala216Gly + Tyr217Glu  
Asp 60Glu + Gly 97Asp + Trp106Asn + Ser159Glu  
Thr 66Asp + Leu 96Glu + Phe189Gly + Gly215Asp  
Asn 62Glu + Thr 66Asp + Tyr104Pro + Gly166Asp  
15 Asn 61Ser + Ala 98Asp + Asn155Asp + Ser188Glu  
Gly100Glu + Tyr104Glu + Ser130Glu + Asn155Gln  
Asp 60Glu + Leu126Asn + Gln206Glu + Lys213Asp  
Ala 98Glu + Gly154Pro + Glul56Asp + Ser188Glu  
Gly128Gln + Ala133Glu + Ala187Glu + Ser191Asp  
20 Ser101Glu + Gly154Asn + Gly211Glu + Tyr214Glu  
Ser132Asp + Asn155Asp + Thr158Glu + Ala216Thr  
Asn 61Glu + Asn155Asp + Ala187Asp + Asn212Gln  
Gln103Glu + Gly160Asn + Gln206Glu + Asn218Glu  
Gln 59Glu + Gly100Glu + Thr164Pro + Gly211Asp  
25 Ser 63Glu + Ser101Asp + Gly131Ser + Val203Pro  
Gln 59Asp + Thr 66Asp + Tyr104Val + Ala133Asp  
Ser 63Glu + Ser101Glu + Ala133His + Ala216Glu  
Asp 60Glu + Val 95Ala + Lys213Glu + Tyr217Ala  
Trp106Met + Ser191Glu + Lys213Glu + Gly219Glu  
30 Ser 63Asp + Gly160Asp + Lys213Asp + Ala216His  
Gly102Asp + Gly157Asn + Ser162Glu + Ser191Glu  
Gln 59Ser + Ser105Asp + Ser162Asp + Ser191Asp  
Gly127Pro + Ser162Glu + Ser191Glu + Asn212Asp  
Ser 63Asp + Ser105Asp + Ser132Asp + Ala216His  
35 Thr 66Gln + Gly128Glu + Glul56Asp + Ala216Asp  
Gly128Asp + Gly157Asn + Pro210Gln + Thr220Glu  
Glul56Asp + Gln206Glu + Lys213Glu + Ala216Asn  
Asp 99Glu + Gly157Pro + Gln206Asp + Lys213Glu  
Ser163Asp + Gln206Asp + Lys213Glu + Tyr217Ala  
40 Gly154Glu + Ser163Glu + Pro210Gln + Tyr217Asp  
Gly154Asp + Gly157Asn + Ser163Asp + Ser204Glu  
Gly154Ser + Gly157Asp + Lys213Glu + Ala216Glu  
Gly157Ser + Thr158Glu + Lys213Asp + Ala216Asp  
Ser101Glu + Gly154Pro + Lys213Asp + Ala216Glu  
45 Gly100Asp + Lys213Glu + Ala216Asp + Tyr217Leu  
Asn 62Ser + Thr158Glu + Ser204Asp + Thr220Asp  
Thr 66Asn + Ile107Val + Lys213Asp + Tyr217Asp  
Gly157Asn + Pro201Gln + Lys213Glu + Tyr217Asp  
Gly127Glu + Thr158Pro + Ala187Asp + Ser204Glu  
50 Asp 99Glu + Ala133Gly + Ser188Glu + Thr220Glu

5 Asp 60Glu + Ser188Glu + Gln206Ser + Asn218Glu  
Gln 59Asp + Leu 96Glu + Gly131Gln + Ser132Asp  
Ser101Glu + Pro129Asp + Thr158Asn + Val203Ser  
Ser 63Glu + Ser163Asp + Ala216Asp + Tyr217Gln  
Gly102Gln + Gly160Glu + Ser191Glu + Lys213Glu  
Val 95Glu + Asp 99Glu + Gly215Glu + Asn218Gln  
Ser105Glu + Ala133Glu + Val203Glu + Asn218Gln  
Gln103Asp + Ser132Glu + Ser162Glu + Gln206Ser  
10 Asp 60Glu + Ser101Asp + Thr164Gly + Lys213Asp  
Gln 59Asp + Asp 99Glu + Gln103Asn + Ala187Pro  
Asp 60Glu + Ser159Asp + Tyr167Leu + Ser188Asp  
Asn 62Glu + Ser163Glu + Gly211Glu + Ala216His  
Asn 62Glu + Ser132Asp + Pro210Gly + Gly211Glu  
Gly102Asn + Ser162Asp + Gln206Asp + Gly219Asp  
15 Ser188Asp + Ser204Asp + Tyr217Leu + Thr220Gln  
Ser 63Glu + Gly166Gln + Ala216Thr + Asn218Glu  
Gln103Glu + Gly131Glu + Tyr217Thr + Thr220Glu  
Asp 60Glu + Phe189His + Asn212Glu + Ala216Asp  
Asn155Gln + Gly215Glu + Tyr217Pro + Gly219Asp  
20 Gly102Asn + Leu126Glu + Ser130Glu + Lys213Asp  
Ala 98Asp + Gly166Glu + Pro210Asp + Tyr214Gln  
Asn 62Glu + Asn155Ser + Lys213Asp + Tyr217Leu  
Asp 60Glu + Ser105Glu + Lys213Glu + Thr220Gln  
Asp 60Glu + Gln206Ser + Lys213Asp + Asn218Asp  
25 Ser 63Glu + Gly 97Gln + Gln103Asp + Gln206Asp  
Ser 63Glu + Val 95Ala + Ser130Asp + Gln206Asp  
Ser 63Asp + Ile107Met + Ser191Asp + Gln206Asp  
Pro129Asn + Ser130Asp + Lys213Glu + Tyr217Glu  
Pro129Asn + Ser191Glu + Lys213Asp + Tyr217Glu  
30 Gly 97Gln + Gly102Asp + Pro129Glu + Phe189Gln  
Gln 59Asn + Ser162Glu + Phe189Asp + Ser204Asp  
Gly127Pro + Gly128Glu + Phe189Glu + Ser204Asp  
Leu 96Pro + Ser105Asp + Ser130Glu + Ala133Gly  
Tyr167His + Ser191Glu + Asn212Glu + Asn218Asp  
35 Asn 61Glu + Thr158Gln + Lys213Asp + Tyr217Asn  
Gln 59Asp + Gly157Asp + Gln206Ser + Asn218Asp  
Gly154Ser + Ser163Glu + Ser188Glu + Ser204Asp  
Leu 96Asn + Ser130Asp + Ser188Asp + Ser204Glu  
Ile107Asp + Ser188Asp + Ser204Asp + Gln206Asn  
40 Gln206Glu + Ala216Gly + Tyr217Leu + Thr220Asp  
Gly102Glu + Leu126Cys + Ser130Glu + Tyr214Asp  
Asn 62Glu + Gly160Asp + Lys213Glu + Ala216Gly  
Ser101Asp + Trp106Met + Gly154Asp + Ser162Asp  
Asp 60Glu + Gly102Glu + Gln206Asn + Ala216Asp  
45 Glu156Asp + Gln206Ser + Pro210Asp + Tyr217Asp  
Pro129Glu + Ser159Asp + Gln206Glu + Tyr217Pro  
Pro129Asp + Ser159Glu + Lys213Asp + Tyr217His  
Ser105Glu + Trp106Leu + Gly127Glu + Ser163Glu  
Ser101Asp + Ala133Gln + Ser191Asp + Val203Asp  
50 Ser 63Glu + Ser130Asp + Tyr217Gln + Gly219Asp

	Gly131Asp + Ser163Asp + Gly166Asn + Ser204Asp
	Ile107Asp + Gln206Ser + Asn212Glu + Ala216Asp
	Leu126Gly + Ser130Asp + Gly154Asn + Asn218Asp
5	Gln 59Asp + Ser105Asp + Gly166Gln + Ser204Asp
	Asn 61Asp + Ser105Glu + Ala187Gln + Ala216Gly
	Ser105Asp + Phe189Ile + Lys213Glu + Gly219Gln
	Ser 63Glu + Gly131Gln + Ser204Glu + Gly219Asn
	Gly157Pro + Thr164Glu + Gln206Asn + Lys213Asp
10	Leu 96Ile + Ser101Asp + Gln206Glu + Tyr214Ala
	Thr 66Gln + Leu 96Met + Tyr167Glu + Ser188Glu
	Tyr104Cys + Gly160Asp + Ile205Pro + Ala216Glu
	Asp 60Glu + Ser130Asp + Pro201Gln + Ala216Gly
	Ile107Asp + Ser191Asp + Gln206Asp + Ala216Thr
15	Gln 59Asp + Val 95Asn + Ser101Glu + Ser163Glu
	Val 95Gln + Tyr104Cys + Lys213Glu + Asn218Asp
	Asn 62Asp + Gly 97Asn + Ala 98Ser + Ser162Glu
	Gln103Glu + Ser204Asp + Gln206Asn + Ala216Pro
	Ser101Asp + Ser162Asp + Gly166Ser + Tyr217Thr
20	Leu126Ile + Gly128Asp + Pro210Ser + Asn218Glu
	Gly100Glu + Gly160Ser + Gly166Glu + Ala216Thr
	Gln103Asn + Ser132Asp + Ser163Glu + Ser188Asp

TABLE 25

Multi-loop Quintuple Mutation Variants	
25	Val 95Gln + Tyr104Cys + Gly127Gln + Lys213Glu + Ala216Pro
	Asn 61Ser + Leu 96His + Gly157Pro + Val203Asp + Ala216Gly
	Leu 96Gln + Gly127Gln + Glu156Asp + Tyr214Ala + Thr220Asn
	Gly100Gln + Tyr167Cys + Ser188Glu + Val203Gln + Ala216His
	Asn 62Ser + Trp106Gly + Ser132Asp + Ala187Ser + Phe189Ser
30	Thr 66Ser + Gly127Gln + Pro201Asn + Ala216Thr + Gly219Asp
	Gly 97Asn + Gly154Pro + Gln206Asn + Pro210Glu + Gly211Pro
	Pro129Gly + Ser132Glu + Thr158Asn + Val165Thr + Gln206Asn
	Gly 65Ser + Gly 97Gln + Gly128Ser + Lys213Asp + Gly219Gln
	Leu 96Met + Gln103Asn + Ala133Ser + Gly154Pro + Gly219Pro
35	Asn 61Gln + Trp106Ala + Gly211Pro + Asn218Asp + Gly219Asn
	Thr 66Gly + Tyr104Ile + Gly211Glu + Gly215Pro + Ala216Gly
	Leu126Ile + Ser130Asp + Gly154Asn + Asn212Ser + Tyr217Thr
	Leu126Val + Gln206Ser + Pro210Gly + Gly215Glu + Ala216Pro
	Leu 96Asn + Leu126Pro + Lys213Asp + Ala216Ser + Tyr217His
40	Trp106Asn + Gly127Ser + Ser161Glu + Gln206Asn + Gly219Asn
	Ser101Glu + Gly102Gln + Ile107Gln + Val165Gln + Val203Ala
	Asp 60Glu + Ala 98Gly + Ile107Ser + Gly157Ser + Thr164Ser
	Pro129Glu + Gly160Pro + Gly166Asn + Ala187Pro + Gly202Ser
	Leu 96Ile + Tyr167Thr + Ser188Asp + Val203His + Gln206Ser
45	Asn 61Gln + Val 95Asp + Gly102Asn + Gly131Asn + Ala187Asn
	Gly160Asn + Val203Thr + Pro210Glu + Asn218Gln + Thr220Gln
	Gly128Asn + Asn155Glu + Gly166Gln + Ala216Gly + Thr220Gly
	Gly 65Ser + Val 95Met + Gly100Asn + Gly131Asp + Tyr214Gly
	Tyr104Gly + Pro129Ser + Ser163Glu + Gln206Ser + Gly219Ser

Asn 61Ser + Val 95Gln + Ser204Asp + Pro210Gly + Ala216Gln  
 Gly 65Gln + Gly 97Pro + Ser130Glu + Gly154Ser + Pro210Asn  
 Trp106Ser + Gly128Asn + Ser159Glu + Pro201Ser + Tyr217Val  
 Leu 96Met + Leu126Asn + Asn155Gln + Ser188Glu + Gly202Gln  
 5 Gly100Glu + Thr158Gln + Thr164Asn + Gln206Asn + Ala216Thr  
 Asn 62Glu + Leu 96Ile + Gly 97Ser + Gly211Ser + Gly219Ser  
 Gly102Asp + Tyr167Ala + Pro210Gly + Ala216Thr + Tyr217Met  
 Ser132Glu + Thr158Pro + Phe189Thr + Ala200Gln + Tyr214Ala  
 Ala 98Pro + Trp106Pro + Gly160Pro + Ala216Asn + Tyr217Asp  
 10 Gly127Pro + Ala133Asn + Thr164Glu + Gly211Gln + Tyr214Thr  
 Gly100Asn + Trp106Pro + Gly127Ser + Lys213Glu + Tyr214Ala  
 Gly157Asn + Ser204Asp + Gln206Asn + Tyr217Val + Gly219Pro  
 Leu 96Thr + Gly131Asp + Ala133Thr + Gln206Asn + Ala216Gly  
 Gly100Ser + Tyr104Ala + Thr164Asp + Gly211Gln + Thr220Ser  
 15 Ser101Asp + Pro129Ser + Phe189Val + Pro201Asn + Ala216Ser  
 Thr 66Gly + Gly102Asn + Tyr104His + Trp106Thr + Ala187Asn  
 Thr 66Asn + Gly102Glu + Trp106Gly + Gly166Ser + Ala216Thr  
 Gly128Gln + Gly154Asn + Tyr167Gly + Tyr217Leu + Asn218Glu  
 Ala133Ser + Gly157Ser + Phe189Thr + Gly202Asn + Asn212Glu  
 20 Tyr104Ser + Thr158Gly + Thr164Glu + Gln206Asn + Ala216Pro  
 Gln 59Asn + Gln103Asn + Thr164Gly + Ala187Pro + Thr220Asp  
 Gly 97Gln + Gly102Asp + Gly127Ser + Phe189Gln + Tyr217Leu  
 Thr 66Asn + Gln206Glu + Tyr214Ile + Ala216Thr + Tyr217Cys  
 Asp 60Glu + Thr 66Gly + Leu 96Gly + Ala216His + Tyr217Asn  
 25 Ile107Asp + Gly160Asn + Val203Pro + Gly211Pro + Gly219Asn  
 Val 95Ser + Trp106Cys + Val165Gln + Pro210Gln + Tyr217Glu  
 Trp106Thr + Thr158Ser + Thr164Pro + Ser204Glu + Thr220Pro  
 Gly128Pro + Ala187Ser + Gln206Asn + Asn212Ser + Gly215Asp  
 Trp106Gln + Leu126Gly + Thr164Ser + Val203Gln + Asn218Asp  
 30 Asp 60Glu + Val 95Gln + Leu126Pro + Gly157Asn + Val203Thr  
 Gln 59Asn + Trp106Cys + Ala200Thr + Gly211Gln + Ala216Gln  
 Asn 62Ser + Ile107Gly + Leu126Cys + Pro210Glu + Thr220Gly  
 Asn 62Gln + Thr158Glu + Val203Ser + Gly215Ser + Ala216Thr  
 Gln 59Asn + Asp 60Glu + Trp106Phe + Gly154Gln + Thr208Pro  
 35 Thr 66Ser + Asn155Gln + Val203Gln + Gln206Glu + Tyr217His  
 Gly128Pro + Phe189Met + Val203Gly + Ser204Glu + Ala216Glu  
 Gln 59Ser + Asn 62Ser + Leu 96Gly + Ser204Glu + Asn218Asp  
 Gln103Ser + Gly128Gln + Ser204Glu + Gly211Asn + Asn218Glu  
 Gly 97Pro + Pro129Gln + Gly157Asn + Ser204Asp + Asn218Glu  
 40 Leu126Asn + Thr158Gln + Val165Met + Gly211Glu + Lys213Glu  
 Gly157Ser + Ser204Glu + Gln206Asp + Tyr217Cys + Thr220Gly  
 Ala133Thr + Phe189Ser + Ser204Asp + Gln206Asp + Tyr214Ile  
 Gly100Gln + Gly154Asn + Ser204Glu + Gln206Asp + Tyr217Thr  
 Gly127Asp + Gly128Glu + Gly154Glu + Gly157Asn + Phe189Ser  
 45 Gly100Gln + Trp106Thr + Ser130Asp + Tyr167Glu + Tyr217Thr  
 Glu156Asp + Thr158Asp + Tyr167Gly + Pro201Gln + Gly215Ser  
 Gly157Gln + Val203Asp + Ser204Asp + Ala216Pro + Gly219Asp  
 Leu126Gly + Pro129Glu + Gly131Glu + Tyr167Met + Thr220Gln  
 Leu 96Ser + Ser130Asp + Gly166Glu + Ala216Gln + Tyr217Ile  
 50 Asn155Glu + Gly160Asn + Gly166Glu + Tyr217Cys + Thr220Asp

Asn 62Asp + Gly 97Gln + Trp106Gly + Pro210Asp + Asn212Gln  
 Val 95Asp + Tyr104Glu + Leu126Ser + Asn155Gln + Gln206Ser  
 Gly154Glu + Thr158Asp + Phe189Glu + Gly215Asn + Tyr217Met  
 Ile107Leu + Gly154Asp + Gly157Glu + Val203His + Gly219Glu  
 5 Trp106Ile + Asn155Ser + Ser159Asp + Ser191Glu + Ala216Thr  
 Gly100Asp + Leu126Asp + Gly127Ser + Pro129Gln + Thr220Ser  
 Ala133Pro + Gln206Glu + Tyr214Ala + Asn218Glu + Gly219Ser  
 Thr 66Gly + Ser101Glu + Gly102Asn + Leu126Glu + Ala216Pro  
 Gly100Glu + Gly102Glu + Tyr104Glu + Asn155Gln + Val203Ala  
 10 Leu126His + Ala187Glu + Val203Glu + Gln206Asp + Asn218Glu  
 Asp 60Glu + Leu 96Asn + Pro129Gln + Gly211Glu + Tyr217Met  
 Leu 96Cys + Ile107Ala + Ala133Pro + Gly157Asp + Gly160Asp  
 Ser 63Asp + Thr158Gly + Gln206Asp + Tyr214Asp + Tyr217Asp  
 Gln 59Asp + Asn 62Asp + Gly100Glu + Phe189Tyr + Tyr214Met  
 15 Ser101Glu + Gly127Glu + Ala187Gln + Gln206Asn + Tyr217Ile  
 Asn 62Asp + Ser 63Glu + Gly100Asp + Gly131Asn + Lys213Glu  
 Asp 60Glu + Gly 97Asp + Ala 98Glu + Phe189His + Gly211Glu  
 Asp 60Glu + Val 95Glu + Asp 99Glu + Ser101Asp + Val165Thr  
 Gly102Gln + Gly154Glu + Asn155Glu + Ser191Asp + Gln206Asp  
 20 Asn 61Ser + Thr 66Ser + Leu126Glu + Asn155Glu + Gly157Asp  
 Pro129Asn + Ala133Gln + Phe189Ile + Gln206Glu + Lys213Glu  
 Asn 61Ser + Gln206Asp + Lys213Glu + Tyr217Ala + Gly219Asn  
 Gln 59Asn + Gly128Asn + Ala200Thr + Gln206Glu + Lys213Glu  
 Phe189Gln + Val203Gly + Gln206Asp + Lys213Asp + Tyr217Pro  
 25 Ala 98His + Gly154Glu + Ser163Asp + Val203Met + Tyr217Met  
 Leu 96Met + Pro129Gly + Gly154Glu + Ser163Glu + Tyr217Ser  
 Gly 97Pro + Ser204Glu + Lys213Asp + Ala216Glu + Gly219Ser  
 Val165Ser + Lys213Glu + Tyr214Cys + Ala216Glu + Tyr217Pro  
 Ser191Glu + Ser204Glu + Gln206Asp + Tyr214His + Ala216Asp  
 30 Gly102Pro + Asn155Asp + Ala216Glu + Tyr217His + Asn218Glu  
 Asn155Asp + Gly215Pro + Ala216Glu + Tyr217Ser + Asn218Glu  
 Gly160Ser + Ser204Glu + Gln206Glu + Lys213Glu + Ala216Ser  
 Ala 98Thr + Ala187Ser + Ser204Glu + Gln206Glu + Lys213Asp  
 Gly127Pro + Ser204Glu + Gln206Glu + Lys213Glu + Tyr217Ala  
 35 Leu126Met + Pro129Glu + Ser163Glu + Phe189Thr + Asn218Ser  
 Ser101Asp + Ser204Asp + Gln206Glu + Ala216Asn + Tyr217Glu  
 Val 95Ala + Tyr167Asp + Ser204Glu + Gln206Glu + Tyr217Glu  
 Asn155Glu + Glu156Asp + Thr164Asp + Ser204Glu + Tyr214Thr  
 Trp106Pro + Gly127Asp + Ser130Asp + Asn155Asp + Gly219Gln  
 40 Pro129Ser + Ser204Asp + Gln206Glu + Pro210Asp + Asn218Glu  
 Tyr104Val + Leu126Asp + Gly157Asp + Ser163Asp + Thr164Asp  
 Leu 96Asp + Gly 97Asp + Gln103Asp + Tyr217Cys + Gly219Asp  
 Ser159Glu + Asn212Gln + Lys213Glu + Gly215Asp + Ala216Glu  
 Gln 59Asp + Asn 62Glu + Ser 63Glu + Pro129Ser + Asn155Asp  
 45 Gln103Ser + Tyr104Ala + Val203Asp + Gln206Asp + Lys213Glu  
 Val 95Glu + Glu156Asp + Gly157Asp + Tyr214Gly + Thr220Asp  
 Val 95Glu + Gly215Glu + Ala216Glu + Tyr217Leu + Gly219Ser  
 Ser 63Asp + Gly160Asp + Ser161Glu + Val203Ser + Tyr217Cys  
 Gly160Asp + Ser161Asp + Tyr167Met + Ser204Asp + Tyr217Ala  
 50 Leu 96His + Trp106Asp + Gln206Asn + Asn218Asp + Gly219Asp

Gly100Glu + Ser101Asp + Trp106Met + Ser162Asp + Thr164Pro  
 Ser105Glu + Ala187Ser + Val203Glu + Ser204Asp + Ala216Gly  
 Asp 60Glu + Trp106Asn + Val203Glu + Ser204Glu + Ala216Gln  
 Gln103Asp + Ser163Glu + Thr164Glu + Pro201Gln + Ala216Pro  
 5 Val 95Gln + Gly100Asn + Glul56Asp + Gly157Asp + Lys213Glu  
 Thr158Asp + Ser159Asp + Ser204Glu + Gly215Asn + Tyr217Cys  
 Ser105Asp + Trp106Glu + Thr164Asn + Ala216Asp + Gly219Ser  
 Gln 59Glu + Asp 60Glu + Tyr104Asn + Ser191Glu + Pro201Gln  
 Gln103Asp + Ser161Glu + Ser162Asp + Gln206Ser + Tyr217His  
 10 Ala 98Asp + Asp 99Glu + Ser105Glu + Thr164Gln + Ala187Ser  
 Gly154Asp + Asn155Asp + Ser204Glu + Ala216Gln + Tyr217Ala  
 Asn 61Glu + Tyr104Ser + Gln206Glu + Ala216Glu + Tyr217Cys  
 Gly157Ser + Thr158Glu + Gln206Asp + Lys213Asp + Ala216Asp  
 Val 95Thr + Gly157Glu + Ser188Glu + Ser204Glu + Ala216Asp  
 15 Tyr104His + Asn155Glu + Gly157Asn + Tyr167Glu + Gly202Ser  
 Gly128Asp + Gly157Asn + Pro210Gln + Asn218Glu + Thr220Glu  
 Asn 62Glu + Val 95Ala + Gly100Asp + Lys213Glu + Tyr217His  
 Gly166Asp + Gln206Ser + Gly215Pro + Tyr217Asp + Gly219Asp  
 Ser130Asp + Ser163Asp + Tyr167Ser + Ser191Asp + Tyr217Met  
 20 Gly 97Pro + Ser132Asp + Thr158Gly + Ser204Glu + Ala216Asp  
 Gly154Asp + Ser191Asp + Lys213Asp + Tyr214Ala + Tyr217Asn  
 Asn 61Gln + Ile107His + Ser204Glu + Lys213Glu + Asn218Glu  
 Gln 59Asp + Ala 98Glu + Gly102Asp + Ser105Glu + Leu209Thr  
 Ala133Gly + Gly154Asp + Gln206Glu + Gly215Glu + Thr220Gln  
 25 Gly154Asn + Gly160Ser + Gly166Glu + Gln206Asp + Gly215Asp  
 Leu 96Glu + Ala 98Asn + Tyr167Asn + Gln206Glu + Gly215Glu  
 Ser162Glu + Thr164Glu + Thr208Gln + Ala216Asp + Tyr217Glu  
 Val 95Asp + Ile107Asp + Tyr167His + Ser188Glu + Thr220Asn  
 Gly154Glu + Gly166Asp + Lys213Asp + Ala216Ser + Tyr217Cys  
 30 Gly 97Glu + Asp 99Glu + Glul56Asp + Tyr167Ala + Ala216Pro  
 Thr 66Gly + Gln103Asp + Trp106Glu + Gly128Asn + Ser162Asp  
 Gln103Glu + Ser105Glu + Thr158Ser + Leu209Thr + Lys213Glu  
 Thr 66Gln + Thr164Asp + Val203His + Gly211Glu + Lys213Glu  
 Pro129Asn + Gly131Gln + Thr164Glu + Gly211Glu + Lys213Asp  
 35 Ser159Asp + Ser162Glu + Gln206Ser + Pro210Glu + Tyr214Ala  
 Asp 99Glu + Ser101Asp + Gly131Asn + Lys213Glu + Gly215Ser  
 Gln103Glu + Tyr104Gly + Thr164Pro + Pro210Asp + Asn212Glu  
 Asn 62Ser + Ser132Asp + Gly160Glu + Ser162Glu + Ala216His  
 Gly160Glu + Ser162Asp + Tyr167Ile + Ser204Glu + Gly219Ser  
 40 Asp 60Glu + Ser 63Asp + Ser130Glu + Gly202Gln + Gly215Ser  
 Gly154Glu + Glul56Asp + Pro210Glu + Lys213Asp + Asn218Gln  
 Ser105Asp + Trp106Gly + Gly127Asp + Gly154Asp + Val165Gln  
 Asn 62Glu + Gly100Glu + Gly157Asn + Gly166Glu + Tyr217Leu  
 Asn 62Asp + Pro129Gly + Ala133Gly + Ser204Asp + Gln206Asp  
 45 Asp 60Glu + Gly100Asn + Ser204Asp + Gln206Glu + Pro210Ser  
 Ser162Glu + Thr164Glu + Val203Thr + Ser204Asp + Asn212Ser  
 Gly 97Glu + Ser130Glu + Tyr167Asp + Tyr217Val + Gly219Ser  
 Gly128Glu + Ser163Glu + Gly166Glu + Gln206Glu + Ala216Ser  
 Asp 60Glu + Asn 61Glu + Ala187Gly + Lys213Glu + Ala216Glu  
 50 Gly 97Asp + Ser101Asp + Tyr104Glu + Ser161Glu + Tyr217Val

Ser 63Glu + Ile107Gln + Gln206Asp + Ala216Asp + Thr220Glu  
 Ser130Glu + Ser132Glu + Gly160Asp + Ala216Gln + Thr220Gly  
 Val 95Glu + Ser130Asp + Ser132Glu + Ala200Gly + Tyr217His  
 Thr 66Gly + Gly100Glu + Gln103Asp + Ser132Asp + Tyr217Asn  
 5 Asp 60Glu + Gly128Glu + Gln206Asn + Pro210Glu + Ala216Gln  
 Leu126Val + Thr158Glu + Val203Met + Lys213Asp + Gly215Glu  
 Asp 99Glu + Ser159Glu + Thr164Glu + Tyr167Leu + Gln206Ser  
 Val 95Asp + Pro129Asn + Thr164Gln + Ala216Glu + Asn218Glu  
 Gly154Asp + Ala187Gly + Gly215Asp + Tyr217Thr + Asn218Glu  
 10 Asn 62Glu + Gly 97Asp + Gly100Asn + Ser204Glu + Tyr217Cys  
 Asn 62Glu + Gly 97Asp + Glu156Asp + Val203Cys + Ala216Gly  
 Asn 62Asp + Gly 97Asp + Ser204Asp + Tyr214Leu + Tyr217Leu  
 Glu156Asp + Ser163Asp + Gln206Ser + Gly215Asp + Ala216Asp  
 Ser159Glu + Ser163Glu + Phe189His + Ser204Glu + Tyr217Ser  
 15 Gly100Pro + Asn155Gln + Ser159Asp + Ser163Asp + Ser204Glu  
 Gly102Asp + Ala187Asp + Ser188Asp + Val203His + Ser204Asp  
 Asp 99Glu + Thr158Asp + Ser162Asp + Val203Met + Ala216Thr  
 Val 95Cys + Gly 97Pro + Ser163Glu + Ser191Asp + Ser204Asp  
 Leu 96Glu + Asp 99Glu + Ser159Glu + Gln206Asn + Ala216Thr  
 20 Gly127Pro + Ser162Glu + Ser191Glu + Gly211Glu + Asn212Asp  
 Ser 63Glu + Ser191Asp + Gln206Asp + Ala216Asp + Tyr217Gln  
 Ser 63Glu + Phe189Ile + Val203Met + Gln206Asp + Gly211Glu  
 Trp106Tyr + Phe189Asp + Pro210Asp + Lys213Glu + Asn218Glu  
 Ser191Glu + Gln206Glu + Ala216Gly + Tyr217Leu + Thr220Asp  
 25 Val 95Gly + Thr158Asp + Ser161Asp + Ala187Pro + Asn218Asp  
 Thr 66Glu + Gly166Glu + Phe189Val + Ser191Glu + Gly219Ser  
 Asp 60Glu + Asp 99Glu + Gln206Glu + Gly211Pro + Ala216Glu  
 Asn 61Asp + Ser 63Asp + Gln103Glu + Lys213Asp + Tyr217Pro  
 Tyr104Glu + Gly128Gln + Ser132Glu + Asn212Asp + Ala216Ser  
 30 Asn 62Asp + Ser204Asp + Gly215Glu + Ala216Gln + Tyr217Leu  
 Asn 61Asp + Gly100Asp + Trp106Ala + Asn212Gln + Lys213Asp  
 Gly127Glu + Gly157Gln + Ser204Asp + Lys213Asp + Ala216Glu  
 Leu 96Glu + Gly 97Ser + Gly100Glu + Gln206Asp + Lys213Asp  
 Asp 60Glu + Leu 96Cys + Gly 97Glu + Ser204Glu + Gly215Asn  
 35 Tyr167Pro + Ser204Asp + Lys213Glu + Ala216His + Gly219Glu  
 Gly 97Ser + Ser105Asp + Asn155Glu + Gly166Asp + Val203Asn  
 Gly102Asn + Gly160Asn + Thr164Glu + Gln206Asn + Thr220Asp  
 Asn 61Ser + Ala 98Asp + Asn155Asp + Ser188Glu + Val203Ser  
 Glu156Asp + Ser204Asp + Gln206Glu + Lys213Glu + Ala216Pro  
 40 Asp 99Glu + Gly157Pro + Ser204Glu + Gln206Asp + Lys213Glu  
 Ser130Asp + Gly160Asn + Ser204Glu + Gln206Asn + Gly215Asp  
 Gly127Glu + Glu156Asp + Ser204Glu + Gln206Asp + Tyr214Pro  
 Ala 98Glu + Asp 99Glu + Trp106Gly + Gly154Asp + Asn218Glu  
 Gln 59Ser + Val 95Glu + Ala 98Asn + Ser105Glu + Gln206Glu  
 45 Gly 97Pro + Gly128Glu + Lys213Asp + Ala216Glu + Asn218Glu  
 Gln103Asp + Ile107Asp + Gly157Pro + Tyr167Glu + Ala216Glu  
 Asp 60Glu + Gln206Glu + Lys213Asp + Gly215Pro + Asn218Glu  
 Ser130Glu + Thr164Glu + Val203Met + Ser204Asp + Gln206Asp  
 Asp 60Glu + Ser 63Glu + Gly154Asp + Gly166Ser + Ser188Asp  
 50 Leu 96His + Ser130Glu + Glu156Asp + Tyr167Glu + Lys213Glu

Gln 59Ser + Glul56Asp + Gly160Glu + Gly211Glu + Lys213Glu  
 Gly127Glu + Asn155Asp + Ala187His + Ala216Glu + Tyr217His  
 Gln103Glu + Gly160Asn + Gln206Glu + Tyr214Gly + Asn218Glu  
 Ser 63Asp + Gly202Pro + Lys213Asp + Gly215Gln + Asn218Asp  
 5 Asp 60Glu + Leu 96Glu + Thr158Gln + Gly166Pro + Gln206Asp  
 Gly 97Asp + Gln103Asp + Phe189Ala + Gln206Ser + Lys213Asp  
 Asn 62Asp + Thr 66Glu + Tyr104Pro + Ser132Asp + Asn212Asp  
 Ala 98Pro + Pro129Asp + Ser130Asp + Lys213Glu + Tyr217Glu  
 Ser 63Asp + Glul56Asp + Gln206Glu + Lys213Glu + Ala216Pro  
 10 Asp 60Glu + Gly102Gln + Ser105Glu + Thr164Gln + Gly211Glu  
 Asp 60Glu + Thr158Gln + Lys213Glu + Ala216Gln + Tyr217Val  
 Ile107Asp + Gly131Asp + Ala216Asp + Tyr217His + Asn218Asp  
 Ser 63Asp + Gly100Glu + Gln103Asp + Gln206Asn + Gly219Asp  
 Asn155Glu + Gly157Glu + Gln206Asn + Pro210Asp + Ala216Glu  
 15 Ser 63Asp + Ile107Met + Pro129Asn + Ser191Asp + Gly219Glu  
 Ser 63Asp + Val 95Ala + Asp 99Glu + Leu126Thr + Ser163Asp  
 Thr 66Glu + Gly100Gln + Gln103Asp + Lys213Asp + Ala216Asn  
 Thr158Asp + Ser161Asp + Gln206Asp + Tyr217Cys + Gly219Asp  
 Ser 63Glu + Ser162Asp + Ala187Gln + Gly211Asn + Lys213Asp  
 20 Gly 97Ser + Ser101Asp + Val203Cys + Tyr214Glu + Tyr217Asp  
 Val 95Glu + Asp 99Glu + Ser204Asp + Gly215Glu + Asn218Gln  
 Gln 59Glu + Thr 66Asp + Ser163Asp + Pro201Gln + Gly215Glu  
 Ala 98His + Ser101Glu + Gly166Gln + Ser188Asp + Val203Asp  
 Ser 63Asp + Gly160Asp + Val203Ala + Ser204Asp + Gln206Glu  
 25 Gly127Glu + Ser162Glu + Ser163Glu + Lys213Asp + Ala216His  
 Ser162Asp + Ala187Glu + Pro201Gln + Gln206Asp + Tyr217Glu  
 Gly157Glu + Phe189Tyr + Val203Glu + Ser204Glu + Lys213Glu  
 Gly160Glu + Ser161Asp + Tyr167Glu + Gly202Asn + Gln206Glu  
 Asp 60Glu + Ser159Asp + Thr164Glu + Phe189His + Lys213Glu  
 30 Tyr104Cys + Ser162Glu + Lys213Glu + Asn218Asp + Gly219Glu  
 Tyr104Asp + Gly128Asn + Ser130Asp + Gly157Ser + Ser204Glu  
 Ser132Glu + Gly157Ser + Ser163Asp + Asn212Asp + Lys213Glu  
 Gly 97Asp + Ala 98Asp + Pro129Glu + Tyr167Leu + Gln206Asp  
 Ser101Glu + Thr158Gln + Ala187Glu + Ser188Glu + Gln206Glu  
 35 Asp 99Glu + Gly100Asp + Asn155Asp + Gly166Gln + Ser204Glu  
 Ser130Glu + Ser161Glu + Ser162Asp + Thr164Asn + Gly211Asp  
 Gln 59Asn + Tyr104Asp + Thr158Asp + Ser191Glu + Asn218Glu  
 Asp 60Glu + Ser101Glu + Ser204Glu + Gln206Ser + Pro210Asp  
 Ser130Asp + Ser159Asp + Ser163Glu + Pro210Gln + Tyr217Asp  
 40 Asn 61Asp + Gly100Asp + Trp106Pro + Gly128Glu + Tyr217Asp  
 Gly102Pro + Gly131Asp + Ser188Asp + Ser204Glu + Gln206Glu  
 Glul56Asp + Ser204Asp + Gln206Asp + Asn212Asp + Ala216His  
 Thr 66Pro + Gln103Asp + Glul56Asp + Ser191Glu + Gln206Asp  
 Gly131Pro + Phe189Leu + Ser191Glu + Gln206Glu + Lys213Glu  
 45 Ala 98Glu + Gly157Ser + Gln206Asp + Lys213Asp + Gly215Gln  
 Tyr104Leu + Thr158Glu + Gly202Ser + Gln206Glu + Lys213Glu  
 Ser 63Glu + Ala 98Gln + Gly102Asn + Ser130Asp + Tyr217Glu  
 Thr158Glu + Gly166Asn + Pro210Glu + Lys213Glu + Thr220Glu  
 Trp106Thr + Gly154Ser + Gly157Asp + Lys213Glu + Ala216Glu  
 50 Ala 98Ser + Ala187Glu + Lys213Asp + Gly215Gln + Ala216Asp



Tyr104Pro + Ser159Asp + Gly202Asn + Lys213Glu + Ala216Asp  
Leu126Asn + Asn155Glu + Thr164Asn + Lys213Asp + Ala216Glu  
Ser161Asp + Val203His + Ser204Asp + Gly211Asp + Tyr217Asp  
Asn 61Asp + Ser163Asp + Val203His + Ser204Glu + Tyr217Asp  
5 Val 95Asp + Trp106Glu + Ser161Glu + Ala187Pro + Ser204Asp  
Leu 96Glu + Gly100Asp + Trp106Cys + Ser188Glu + Gln206Asp  
Ser101Glu + Ser204Asp + Gly211Glu + Lys213Asp + Gly215Asn  
Asp 99Glu + Ser159Glu + Ser162Glu + Ser204Asp + Gly219Asn  
Leu 96Ala + Gln103Asp + Leu126Val + Gly128Asp + Ser204Asp  
10 Ala 98Glu + Ser105Glu + Gly154Glu + Glu156Asp + Phe189Pro  
Asn 61Glu + Ser159Glu + Gln206Ser + Pro210Glu + Ala216Glu  
Gly 97Asp + Ser101Asp + Ala133Glu + Gln206Glu + Gly219Pro  
Leu126Ala + Gly131Glu + Ser204Glu + Pro210Asp + Lys213Glu  
Val 95Glu + Ala 98Asn + Gly102Glu + Ser162Asp + Ser204Glu  
15 Asn 61Glu + Gly100Asn + Pro129Asp + Ser163Glu + Asn218Ser  
Gly102Asp + Gly127Ser + Thr158Asp + Gly160Glu + Lys213Glu  
Ser130Asp + Asn155Gln + Thr158Glu + Ser191Asp + Gly215Glu  
Ala133Asp + Ser159Glu + Ser161Asp + Ser204Asp + Ala216Gln  
Ser132Glu + Thr164Asp + Ser204Asp + Gln206Glu + Tyr217Pro  
20 Gly157Glu + Tyr167Asp + Ser204Glu + Gln206Glu + Ala216Asn  
Thr 66Ser + Ser130Glu + Thr158Glu + Ser204Glu + Gln206Glu  
Asp 99Glu + Ser159Glu + Ser204Glu + Gln206Glu + Tyr217Pro  
Thr 66Ser + Ser105Asp + Ser159Glu + Ser204Glu + Gln206Asp  
Asp 60Glu + Gly127Asp + Ser204Glu + Gln206Glu + Tyr214Asn  
25 Ser 63Glu + Ser130Asp + Gln206Asp + Ala216Gly + Asn218Asp  
Pro129Gly + Ser159Glu + Ser188Glu + Phe189Cys + Ser204Asp  
Gly131Asp + Glu156Asp + Ser162Glu + Ala187Pro + Tyr214Gly  
Gly102Asp + Trp106Glu + Ser159Glu + Pro210Gln + Thr220Asp  
Gly131Asp + Ser161Asp + Ser163Asp + Gly166Asn + Ser204Asp  
30 Gln 59Asn + Ser188Asp + Gln206Asp + Gly211Glu + Tyr217Glu  
Ala 98Glu + Gly157Asp + Thr164Asp + Phe189Thr + Lys213Asp  
Gln103Asp + Trp106Tyr + Gly160Asp + Lys213Glu + Gly215Asp  
Val 95Asp + Gly131Gln + Ser159Asp + Ala216Asp + Asn218Asp  
Ser101Asp + Gln103Glu + Ser161Glu + Gln206Glu + Ala216His  
35 Thr 66Glu + Gly128Pro + Gly154Asp + Thr164Asp + Ser204Glu  
Val 95Asp + Gly131Glu + Ser163Asp + Ser191Glu + Gln206Asn  
Val 95Ser + Ala 98Glu + Ser101Asp + Gly131Asp + Phe189Asp  
Asn 62Asp + Leu126His + Gly131Pro + Lys213Glu + Tyr217Asp  
Ser 63Asp + Ser130Glu + Thr158Pro + Ala216Glu + Tyr217Ile  
40 Gln 59Asp + Gly157Asp + Gln206Glu + Tyr214Val + Asn218Asp  
Val 95Glu + Asp 99Glu + Gly215Asp + Ala216Asn + Tyr217Ile  
Ser132Glu + Gly154Gln + Gly157Glu + Ser161Asp + Tyr214Ser  
Ser101Asp + Gly131Pro + Ser188Asp + Ser191Glu + Gln206Glu  
Thr 66Asp + Leu 96Glu + Glu156Asp + Val203His + Gly215Asp  
45 Asn 62Glu + Gly166Gln + Ser188Glu + Gly211Glu + Ala216His  
Ile107Asp + Ala187Asp + Ser191Asp + Gln206Asp + Ala216Thr  
Ser105Asp + Ser159Glu + Ser191Asp + Lys213Asp + Ala216Thr  
Asn155Asp + Ser163Asp + Val165Asn + Gln206Ser + Lys213Glu  
Ser101Glu + Gly131Asn + Asn155Glu + Ala187Glu + Lys213Asp  
50 Gln 59Glu + Gly160Asp + Ser188Asp + Val203Glu + Tyr217Ile

Ala133Asp + Ser161Glu + Thr164Asp + Ser204Asp + Asn218Ser  
 Gln103Glu + Tyr104Cys + Ser161Glu + Thr164Asp + Lys213Glu  
 Ser 63Glu + Gly160Asp + Tyr167Met + Lys213Asp + Asn218Asp  
 Ser101Glu + Leu126Glu + Ser188Glu + Lys213Asp + Ala216Asn  
 5 Asp 60Glu + Leu 96Glu + Gly128Asn + Ser130Glu + Gln206Glu  
 Gln103Ser + Ser130Asp + Ala133Gly + Gln206Glu + Gly219Asp  
 Gly102Asn + Ser162Asp + Gln206Asp + Tyr217Gly + Gly219Asp  
 Thr 66Gln + Asp 99Glu + Gln103Glu + Val203Ser + Tyr217Asp  
 Asp 99Glu + Gln103Asp + Gly157Asn + Lys213Asp + Ala216Gln  
 10 Thr 66Asp + Pro129Asp + Ser159Glu + Lys213Asp + Tyr217His  
 Ser 63Asp + Gly 97Asp + Tyr167Ala + Ser188Asp + Ser204Glu  
 Gly102Pro + Tyr104Ala + Glul56Asp + Tyr167Glu + Ser204Glu  
 Gln 59Glu + Asn 62Gln + Gln103Glu + Gly131Glu + Phe189Leu  
 Asp 60Glu + Ser162Glu + Ala200Gln + Val203Glu + Gly211Asp  
 15 Asp 60Glu + Ile107Glu + Gly157Asp + Gly160Glu + Phe189Ser  
 Ser101Asp + Gly102Ser + Tyr104Glu + Phe189Asp + Lys213Glu  
 Ser101Asp + Ser105Asp + Val203Asp + Ala216His + Tyr217His  
 Ser132Asp + Asn155Glu + Gly211Pro + Lys213Glu + Asn218Asp  
 Gln103Asp + Gly128Asp + Ser163Asp + Ala187Glu + Tyr217Ile  
 20 Leu 96Ile + Gly128Asp + Ser191Glu + Gly202Asn + Gln206Glu  
 Thr 66Glu + Gln103Asp + Ser204Glu + Lys213Asp + Gly219Ser  
 Ala 98Asp + Ser132Asp + Gly166Glu + Pro210Asp + Tyr214Gln  
 Ser 63Glu + Pro129Glu + Val203Met + Lys213Glu + Gly219Asp  
 Gln 59Glu + Gly 97Asp + Gly128Asp + Ser159Glu + Ala216Ser  
 25 Ser 63Glu + Gln103Glu + Ile107Ser + Glul56Asp + Lys213Asp  
 Gly102Asp + Gly157Asn + Ser162Glu + Ser191Glu + Ser204Glu  
 Ser105Asp + Ser162Asp + Ser191Asp + Pro210Gly + Gly211Glu  
 Asp 60Glu + Val 95Glu + Trp106Gly + Pro129Glu + Ser159Asp  
 Ser101Glu + Trp106Asp + Thr164Glu + Ser204Asp + Pro210Ser  
 30 Gln 59Glu + Gly100Gln + Gly157Asp + Gly211Asp + Tyr217Glu  
 Gly 97Asp + Ser130Asp + Gln206Asp + Lys213Asp + Ala216Asn  
 Tyr104Asp + Gly154Asp + Gly160Asn + Ser163Asp + Ser204Glu  
 Ser132Glu + Gly154Glu + Ser163Glu + Pro210Gly + Asn212Asp  
 Leu 96Thr + Ala133Glu + Asn155Glu + Lys213Asp + Ala216Asp  
 35 Asp 60Glu + Asp 99Glu + Leu126Gly + Ser130Asp + Ser162Glu

## II Cleaning Compositions

In another embodiment of the present invention, an effective amount of  
 one or more of the enzyme variants are included in compositions useful for  
 40 cleaning a variety of surfaces in need of proteinaceous stain removal. Such  
 cleaning compositions include detergent compositions for cleaning hard  
 surfaces, unlimited in form (e.g., liquid and granular); detergent compositions for  
 cleaning fabrics, unlimited in form (e.g., granular, liquid and bar formulations);  
 dishwashing compositions (unlimited in form); oral cleaning compositions,  
 45 unlimited in form (e.g., dentifrice, toothpaste and mouthwash formulations);  
 denture cleaning compositions, unlimited in form (e.g., liquid, tablet); and contact

lens cleaning compositions, unlimited in form (e.g., liquid, tablet).

The cleaning compositions also comprise, in addition to the BPN' variants described hereinbefore, one or more cleaning composition materials compatible with the protease enzyme. the term "cleaning composition material", as used  
5 herein, means any liquid, solid or gaseous material selected for the particular type of cleaning composition desired and the form of the product (e.g., liquid, granule, bar, spray, stick, paste, gel), which materials are also compatible with the BPN' variant used in the composition. the specific selection of cleaning composition materials are readily made by considering the surface material to be  
10 cleaned, the desired form of the composition for the cleaning condition during use (e.g., through the wash detergent use). The term "compatible", as used herein, means the cleaning composition materials do not reduce the proteolytic activity of the BPN' variant to such an extent that the protease is not effective as desired during normal use situations. Specific cleaning composition materials  
15 are exemplified in detail hereinafter.

As used herein, "effective amount of enzyme variant" refers to the quantity of enzyme variant necessary to achieve the enzymatic activity necessary in the specific cleaning composition. Such effective amounts are readily ascertained by one of ordinary skill in the art and is based on many factors, such as the  
20 particular enzyme variant used, the cleaning application, the specific composition of the cleaning composition, and whether a liquid or dry (e.g., granular, bar) composition is required, and the like. Preferably the cleaning compositions comprise from about 0.0001% to about 10% of one or more enzyme variants of the present invention, more preferably from about 0.001% to  
25 about 1%, more preferably still from about 0.01% to about 0.1%. Several examples of various cleaning compositions wherein the enzyme variants may be employed are discussed in further detail below. All parts, percentages and ratios used herein are by weight unless otherwise specified.

As used herein, "non-fabric cleaning compositions" include hard surface  
30 cleaning compositions, dishwashing compositions, oral cleaning compositions, denture cleaning compositions and contact lens cleaning compositions.

A. Cleaning Compositions for Hard Surfaces, Dishes and Fabrics

The enzyme variants of the present invention can be used in a variety of detergent compositions where high sudsing and good insoluble substrate  
35 removal are desired. Thus the enzyme variants can be used with various

conventional ingredients to provide fully-formulated hard-surface cleaners, dishwashing compositions, fabric laundering compositions and the like. Such compositions can be in the form of liquids, granules, bars and the like. Such compositions can be formulated as modern "concentrated" detergents which  
5 contain as much as 30%-60% by weight of surfactants.

The cleaning compositions herein can optionally, and preferably, contain various anionic, nonionic, zwitterionic, etc., surfactants. Such surfactants are typically present at levels of from about 5% to about 35% of the compositions.

Nonlimiting examples of surfactants useful herein include the  
10 conventional C<sub>11</sub>-C<sub>18</sub> alkyl benzene sulfonates and primary and random alkyl sulfates, the C<sub>10</sub>-C<sub>18</sub> secondary (2,3) alkyl sulfates of the formulas  $\text{CH}_3(\text{CH}_2)_x(\text{CHOSO}_3^- \text{M}^+)\text{CH}_3$  and  $\text{CH}_3(\text{CH}_2)_y(\text{CHOSO}_3^- \text{M}^+)\text{CH}_2\text{CH}_3$  wherein x and (y+1) are integers of at least about 7, preferably at least about 9, and M is a water-solubilizing cation, especially sodium, the C<sub>10</sub>-C<sub>18</sub> alkyl alkoxy  
15 sulfates (especially EO 1-5 ethoxy sulfates), C<sub>10</sub>-C<sub>18</sub> alkyl alkoxy carboxylates (especially the EO 1-5 ethoxycarboxylates), the C<sub>10</sub>-C<sub>18</sub> alkyl polyglycosides, and their corresponding sulfated polyglycosides, C<sub>12</sub>-C<sub>18</sub> alpha-sulfonated fatty acid esters, C<sub>12</sub>-C<sub>18</sub> alkyl and alkyl phenol alkoxyates (especially ethoxylates and mixed ethoxy/propoxy), C<sub>12</sub>-C<sub>18</sub> betaines and sulfobetaines ("sultaines"),  
20 C<sub>10</sub>-C<sub>18</sub> amine oxides, and the like. The alkyl alkoxy sulfates (AES) and alkyl alkoxy carboxylates (AEC) are preferred herein. (Use of such surfactants in combination with the aforesaid amine oxide and/or betaine or sultaine surfactants is also preferred, depending on the desires of the formulator.) Other conventional useful surfactants are listed in standard texts. Particularly useful  
25 surfactants include the C<sub>10</sub>-C<sub>18</sub> N-methyl glucamides disclosed in US Patent 5, 194,639, Connor et al., issued March 16, 1993, incorporated herein by reference.

A wide variety of other ingredients useful in detergent cleaning compositions can be included in the compositions herein, including other active  
30 ingredients, carriers, hydrotropes, processing aids, dyes or pigments, solvents for liquid formulations, etc. If an additional increment of sudsing is desired, suds boosters such as the C<sub>10</sub>-C<sub>16</sub> alkolamides can be incorporated into the compositions, typically at about 1% to about 10% levels. The C<sub>10</sub>-C<sub>14</sub> monoethanol and diethanol amides illustrate a typical class of such suds  
35 boosters. Use of such suds boosters with high sudsing adjunct surfactants such

as the amine oxides, betaines and sultaines noted above is also advantageous. If desired, soluble magnesium salts such as  $MgCl_2$ ,  $MgSO_4$ , and the like, can be added at levels of, typically, from about 0.1% to about 2%, to provide additionally sudsing.

5       The liquid detergent compositions herein can contain water and other solvents as carriers. Low molecular weight primary or secondary alcohols exemplified by methanol, ethanol, propanol, and isopropanol are suitable. Monohydric alcohols are preferred for solubilizing surfactants, but polyols such as those containing from about 2 to about 6 carbon atoms and from about 2 to  
10       about 6 hydroxy groups (e.g., 1,3-propanediol, ethylene glycol, glycerine, and 1,2-propanediol) can also be used. The compositions may contain from about 5% to about 90%, typically from about 10% to about 50% of such carriers.

15       The detergent compositions herein will preferably be formulated such that during use in aqueous cleaning operations, the wash water will have a pH between about 6.8 and about 11.0. Finished products thus are typically formulated at this range. Techniques for controlling pH at recommended usage levels include the use of buffers, alkalis, acids, etc., and are well known to those skilled in the art.

20       When formulating the hard surface cleaning compositions and fabric cleaning compositions of the present invention, the formulator may wish to employ various builders at levels from about 5% to about 50% by weight. Typical builders include the 1-10 micron zeolites, polycarboxylates such as citrate and oxydisuccinates, layered silicates, phosphates, and the like. Other conventional builders are listed in standard formularies.

25       Likewise, the formulator may wish to employ various additional enzymes, such as cellulases, lipases, amylases and proteases in such compositions, typically at levels of from about 0.001% to about 1% by weight. Various deterative and fabric care enzymes are well-known in the laundry detergent art.

30       Various bleaching compounds, such as the percarbonates, perborates and the like, can be used in such compositions, typically at levels from about 1% to about 15% by weight. If desired, such compositions can also contain bleach activators such as tetraacetyl ethylenediamine, nonanoyloxybenzene sulfonate, and the like, which are also known in the art. Usage levels typically range from about 1% to about 10% by weight.

35       Various soil release agents, especially of the anionic oligoester type,

various chelating agents, especially the aminophosphonates and ethylenediaminedisuccinates, various clay soil removal agents, especially ethoxylated tetraethylene pentamine, various dispersing agents, especially polyacrylates and polyasparatates, various brighteners, especially anionic  
5 brighteners, various suds suppressors, especially silicones and secondary alcohols, various fabric softeners, especially smectite clays, and the like can all be used in such compositions at levels ranging from about 1% to about 35% by weight. Standard formularies and published patents contain multiple, detailed descriptions of such conventional materials.

10 Enzyme stabilizers may also be used in the cleaning compositions. Such enzyme stabilizers include propylene glycol (preferably from about 1% to about 10%), sodium formate (preferably from about 0.1% to about 1%) and calcium formate (preferably from about 0.1% to about 1%).

1. Hard surface cleaning compositions

15 As used herein "hard surface cleaning composition" refers to liquid and granular detergent compositions for cleaning hard surfaces such as floors, walls, bathroom tile, and the like. Hard surface cleaning compositions of the present invention comprise an effective amount of one or more enzyme variants of the present invention, preferably from about 0.001% to about 10%, more preferably  
20 from about .01% to about 5%, more preferably still from about .05% to about 1% by weight of active enzyme of the composition. In addition to comprising one or more of the enzyme variants, such hard surface cleaning compositions typically comprise a surfactant and a water-soluble sequestering builder. In certain specialized products such as spray window cleaners, however, the surfactants  
25 are sometimes not used since they may produce a filmy/streaky residue on the glass surface.

The surfactant component, when present, may comprise as little as 0.1% of the compositions herein, but typically the compositions will contain from about 0.25% to about 10%, more preferably from about 1% to about 5% of surfactant.

30 Typically the compositions will contain from about 0.5% to about 50% of a detergency builder, preferably from about 1% to about 10%.

Preferably the pH should be in the range of about 8 to 12. Conventional pH adjustment agents such as sodium hydroxide, sodium carbonate or hydrochloric acid can be used if adjustment is necessary.

35 Solvents may be included in the compositions. Useful solvents include,

but are not limited to, glycol ethers such as diethyleneglycol monohexyl ether, diethyleneglycol monobutyl ether, ethyleneglycol monobutyl ether, ethyleneglycol monohexyl ether, propyleneglycol monobutyl ether, dipropyleneglycol monobutyl ether, and diols such as 2,2,4-trimethyl-1,3-pentanediol and 2-ethyl-1,3-hexanediol. When used, such solvents are typically present at levels of from about 0.5% to about 15%, preferably from about 3% to about 11%.

Additionally, highly volatile solvents such as isopropanol or ethanol can be used in the present compositions to facilitate faster evaporation of the composition from surfaces when the surface is not rinsed after "full strength" application of the composition to the surface. When used, volatile solvents are typically present at levels of from about 2% to about 12% in the compositions.

The hard surface cleaning composition embodiment of the present invention is illustrated by the following examples.

#### Examples 7-12

##### Liquid Hard Surface Cleaning Compositions

Component	Example No.					
	7	8	9	10	11	12
Ser105Glu	0.05	0.50	0.02	0.03	0.10	0.03
Gly127Gln + Ala216Pro	—	—	—	—	0.20	0.02
Na <sub>2</sub> DIDA*						
EDTA**	—	—	2.90	2.90	—	—
Na Citrate	—	—	—	—	2.90	2.90
NaC <sub>12</sub> Alkyl-benzene sulfonate	1.95	—	1.95	—	1.95	—
NaC <sub>12</sub> Alkylsulfate	—	2.20	—	2.20	—	2.20
NaC <sub>12</sub> (ethoxy)*** sulfate	—	2.20	—	2.20	—	2.20
C <sub>12</sub> Dimethylamine oxide	—	0.50	—	0.50	—	0.50
Na Cumene sulfonate	1.30	—	1.30	—	1.30	—
Hexyl Carbitol***	6.30	6.30	6.30	6.30	6.30	6.30
Water****	balance to 100%					

\*Disodium N-diethyleneglycol-N,N-iminodiacetate

\*\*Na<sub>4</sub> ethylenediamine diacetic acid

\*\*\*Diethyleneglycol monohexyl ether

\*\*\*\*All formulas adjusted to pH 7

In Examples 7-10, the BPN' variants recited in Tables 2-25, among others, are substituted for Ser105Glu, with substantially similar results.

- 5 In Examples 11-12, any combination of the BPN' variants recited in Tables 2-25, among others, are substituted for Gly127Gln + Ala216Pro, with substantially similar results.

#### Examples 13-18

#### Spray Compositions for Cleaning Hard Surfaces and Removing Household Mildew

Component	Example No.					
	13	14	15	16	17	18
Tyr104Ile + Gly215Pro	0.50	0.05	0.60	0.30	0.20	0.30
Asp99Glu	-	-	-	-	0.30	0.10
15 Sodium octyl sulfate	2.00	2.00	2.00	2.00	2.00	2.00
Sodium dodecyl sulfate	4.00	4.00	4.00	4.00	4.00	4.00
Sodium hydroxide	0.80	0.80	0.80	0.80	0.80	0.80
Silicate (Na)	0.04	0.04	0.04	0.04	0.04	0.04
Perfume	0.35	0.35	0.35	0.35	0.35	0.35
20 Water	balance to 100%					

Product pH is about 7.

In Examples 13-16, the BPN' variants recited in Tables 2-25, among others, are substituted for Tyr104Ile + Gly215Pro, with substantially similar results.

- 25 In Examples 17-18, any combination of the BPN' variants recited in Tables 2-25, among others, are substituted for Tyr104Ile + Gly215Pro and Asp99Glu, with substantially similar results.

#### 2. Dishwashing Compositions

- 30 In another embodiment of the present invention, dishwashing compositions comprise one or more enzyme variants of the present invention. As used herein, "dishwashing composition" refers to all forms for compositions for cleaning dishes, including but not limited to, granular and liquid forms. The dishwashing composition embodiment of the present invention is illustrated by the following examples.



**Examples 19-24**  
**Dishwashing Composition**

		Example No.					
Component		19	20	21	22	23	24
5	Glu59Ser + Leu96Gly						
	+ Ser204Glu	0.05	0.50	0.02	0.40	0.10	0.03
	Lys96Gly + Ser204Glu	-	-	-	-	0.40	0.02
	C <sub>12</sub> -C <sub>14</sub> N-methyl- glucamide	0.90	0.90	0.90	0.90	0.90	0.90
10	C <sub>12</sub> ethoxy (1) sulfate	12.00	12.00	12.00	12.00	12.00	12.00
	2-methyl undecanoic acid	4.50	4.50	4.50	4.50	4.50	4.50
	C <sub>12</sub> ethoxy (2) carboxylate	4.50	4.50	4.50	4.50	4.50	4.50
	C <sub>12</sub> alcohol ethoxylate (4)	3.00	3.00	3.00	3.00	3.00	3.00
	C <sub>12</sub> amine oxide	3.00	3.00	3.00	3.00	3.00	3.00
15	Sodium cumene sulfonate	2.00	2.00	2.00	2.00	2.00	2.00
	Ethanol	4.00	4.00	4.00	4.00	4.00	4.00
	Mg <sup>++</sup> (as MgCl <sub>2</sub> )	0.20	0.20	0.20	0.20	0.20	0.20
	Ca <sup>++</sup> (as CaCl <sub>2</sub> )	0.40	0.40	0.40	0.40	0.40	0.40
	Water	balance to 100%					

20 Product pH is adjusted to 7.

In Examples 19-22, the BPN' variants recited in Tables 2-25, among others, are substituted for Gln59SSer + Leu96Gly + Ser204Glu, with substantially similar results.

25 In Examples 23-24, any combination of the BPN' variants recited in Tables 2-25, among others, are substituted for Gln59SSer + Leu96Gly + Ser204Glu and Lys96Gly + Ser204Glu, with substantially similar results.

### 3. Fabric cleaning compositions

30 In another embodiment of the present invention, fabric cleaning compositions comprise one or more enzyme variants of the present invention. As used herein, "fabric cleaning composition" refers to all forms for detergent compositions for cleaning fabrics, including but not limited to, granular, liquid and bar forms. Preferred fabric cleaning compositions are those in the liquid form.

#### a. Granular fabric cleaning compositions

35 The granular fabric cleaning compositions of the present invention contain

an effective amount of one or more enzyme variants of the present invention, preferably from about 0.001% to about 10%, more preferably from about 0.005% to about 5%, more preferably from about 0.01% to about 1% by weight of active enzyme of the composition. In addition to one or more enzyme variants, the granular fabric cleaning compositions typically comprise at least one surfactant, one or more builders, and, in some cases, a bleaching agent.

The granular fabric cleaning composition embodiment of the present invention is illustrated by the following examples.

#### Examples 25-28

Granular Fabric Cleaning Composition				
Component	Example No.			
	25	26	27	28
Ser101Asp	0.10	0.20	0.03	0.05
Thr66Glu	-	-	0.02	0.05
C <sub>13</sub> linear alkyl benzene sulfonate	22.00	22.00	22.00	22.00
Phosphate (as sodium tripolyphosphates)	23.00	23.00	23.00	23.00
Sodium carbonate	23.00	23.00	23.00	23.00
Sodium silicate	14.00	14.00	14.00	14.00
Zeolite	8.20	8.20	8.20	8.20
Chelant (diethylenetriamine-pentaacetic acid)	0.40	0.40	0.40	0.40
Sodium sulfate	5.50	5.50	5.50	5.50
Water	balance to 100%			

In Examples 25-26, the BPN' variants recited in Tables 2-25, among others, are substituted for Ser101Asp, with substantially similar results.

In Examples 27-28, any combination of the BPN' variants recited in Tables 2-25, among others, are substituted for Ser101Asp and Thr66Glu, with substantially similar results.

**Examples 29-32**  
**Granular Fabric Cleaning Composition**

Component		Example No.			
		29	30	31	32
5	Val95Asp + Leu126Ser + Asn155Gln	0.10	0.20	0.03	0.05
	Gly65Ser + Gly102Asn + Val203Glu	-	-	0.02	0.05
	C <sub>12</sub> alkyl benzene sulfonate	12.00	12.00	12.00	12.00
	Zeolite A (1-10 micrometer)	26.00	26.00	26.00	26.00
	2-butyl octanoic acid	4.00	4.00	4.00	4.00
10	C <sub>12</sub> -C <sub>14</sub> secondary (2,3) alkyl sulfate, Na salt	5.00	5.00	5.00	5.00
	Sodium citrate	5.00	5.00	5.00	5.00
	Optical brightener	0.10	0.10	0.10	0.10
	Sodium sulfate	17.00	17.00	17.00	17.00
15	Water and minors	balance to 100%			

In Examples 29-30, the BPN' variants recited in Tables 2-25, among others, are substituted for Val95Asp + Leu126Ser + Asn155Gln, with substantially similar results.

In Examples 31-32, any combination of the BPN' variants recited in  
 20 Tables 2-25, among others, are substituted for Val95Asp + Leu126Ser + Asn155Gln and Gly65Ser + Gly102Asn + Val203Glu, with substantially similar results.

**Examples 33-36**  
**Granular Fabric Cleaning Composition**

		Example No.			
Component		33	34	35	36
5	Ser63Glu	0.10	0.20	0.03	0.05
	Leu96Asn + Lys213Asp	-	-	0.02	0.05
	C <sub>13</sub> linear alkyl benzene sulfonate	22.00	22.00	22.00	22.00
	Phosphate (as sodium tripolyphosphates)	23.00	23.00	23.00	23.00
10	Sodium carbonate	23.00	23.00	23.00	23.00
	Sodium silicate	14.00	14.00	14.00	14.00
	Zeolite	8.20	8.20	8.20	8.20
	Chelant (diethylenetriamine-pentaacetic acid)	0.40	0.40	0.40	0.40
15	Sodium sulfate	5.50	5.50	5.50	5.50
	Water	balance to 100%			

In Examples 33-34, the BPN' variants recited in Tables 2-25, among others, are substituted for Ser63Glu, with substantially similar results.

In Examples 35-36, any combination of the BPN' variants recited in  
 20 Tables 2-25, among others, are substituted for Ser63Glu and Leu96Asn + Lys213Asp, with substantially similar results.

**Examples 37-40**  
**Granular Fabric Cleaning Composition**

		Example No.			
Component		37	38	39	40
5	Asn62Ser + Ser163Asp + Phe189Ser + Ala216Glu	0.10	0.20	0.03	0.05
	Gly97Ser + Trp106Ile + Tyr217Leu	-	-	0.02	0.05
	C <sub>12</sub> alkyl benzene sulfonate	12.00	12.00	12.00	12.00
	Zeolite A (1-10 micrometer)	26.00	26.00	26.00	26.00
10	2-butyl octanoic acid	4.00	4.00	4.00	4.00
	C <sub>12</sub> -C <sub>14</sub> secondary (2,3) alkyl sulfate, Na salt	5.00	5.00	5.00	5.00
	Sodium citrate	5.00	5.00	5.00	5.00
	Optical brightener	0.10	0.10	0.10	0.10
15	Sodium sulfate	17.00	17.00	17.00	17.00
	Water and minors	balance to 100%			

In Examples 37-38, the BPN' variants recited in Tables 2-25, among others, are substituted for Asn62Ser + Ser163Asp + Phe189Ser + Ala216Glu, with substantially similar results.

- 20 In Examples 39-40, any combination of the BPN' variants recited in Tables 2-25, among others, are substituted for Asn62Ser + Ser163Asp + Phe189Ser + Ala216Glu and Gly97Ser + Trp106Ile + Tyr217Leu, with substantially similar results.

**Examples 41-42**  
**Granular Fabric Cleaning Composition**

		Example No.	
Component		41	42
5	Linear alkyl benzene sulphonate	11.4	10.70
	Tallow alkyl sulphate	1.80	2.40
	C <sub>14-15</sub> alkyl sulphate	3.00	3.10
	C <sub>14-15</sub> alcohol 7 times ethoxylated	4.00	4.00
	Tallow alcohol 11 times ethoxylated	1.80	1.80
10	Dispersant	0.07	0.1
	Silicone fluid	0.80	0.80
	Trisodium citrate	14.00	15.00
	Citric acid	3.00	2.50
	Zeolite	32.50	32.10
15	Maleic acid acrylic acid copolymer	5.00	5.00
	Diethylene triamine penta methylene phosphonic acid	1.00	0.20
	Ala98Asp + Ala187Ser	0.30	0.30
	Lipase	0.36	0.40
20	Amylase	0.30	0.30
	Sodium silicate	2.00	2.50
	Sodium sulphate	3.50	5.20
	Polyvinyl pyrrolidone	0.30	0.50
	Perborate	0.5	1
25	Phenol sulphonate	0.1	0.2
	Peroxidase	0.1	0.1
	Minors	Up to 100	Up to 100

Examples 43-44  
Granular Fabric Cleaning Composition

		Example No.	
Component		43	44
5	Sodium linear C <sub>12</sub> alkyl benzene-sulfonate	6.5	8.0
	Sodium sulfate	15.0	18.0
	Zeolite A	26.0	22.0
	Sodium nitrilotriacetate	5.0	5.0
	Polyvinyl pyrrolidone	0.5	0.7
10	Tetraacetylene diamine	3.0	3.0
	Boric acid	4.0	-
	Perborate	0.5	1
	Phenol sulphonate	0.1	0.2
	Gln59Ser + Asn62Ser + Leu96Gly	0.4	0.4
15	+ Ser204Gln		
	Fillers (e.g., silicates; carbonates; perfumes; water)	Up to 100	Up to 100

Example 45  
Compact Granular Fabric Cleaning Composition

		Weight %
20	Component	
	Alkyl Sulphate	8.0
	Alkyl Ethoxy Sulphate	2.0
	Mixture of C <sub>25</sub> and C <sub>45</sub> alcohol 3 and 7 times ethoxylated	6.0
	Polyhydroxy fatty acid amide	2.5
	Zeolite	17.0
	Layered silicate/citrate	16.0
	Carbonate	7.0
	Maleic acid acrylic acid copolymer	5.0
	Soil release polymer	0.4
	Carboxymethyl cellulose	0.4
	Poly (4-vinylpyridine) -N-oxide	0.1
	Copolymer of vinylimidazole and vinylpyrrolidone	0.1
	PEG2000	0.2
	Val95Gln + Tyr104Glu + Gly127Gln + Lys213Glu + Ala216Asp	0.5
	Lipase	0.2

Cellulase	0.2
Tetracetylene diamine	6.0
Percarbonate	22.0
Ethylene diamine disuccinic acid	0.3
Suds suppressor	3.5
Disodium-4,4'-bis (2-morpholino -4-anilino-s-triazin-6-ylamino) stilbene-2,2'-disulphonate	0.25
Disodium-4,4'-bis (2-sulfostyryl) biphenyl	0.05
Water, Perfume and Minors	Up to 100

## Example 46

## Granular Fabric Cleaning Composition

Component	Weight %
Linear alkyl benzene sulphonate	7.6
C <sub>16</sub> -C <sub>18</sub> alkyl sulfate	1.3
C <sub>14-15</sub> alcohol 7 times ethoxylated	4.0
Coco-alkyl-dimethyl hydroxyethyl ammonium chloride	1.4
Dispersant	0.07
Silicone fluid	0.8
Trisodium citrate	5.0
Zeolite 4A	15.0
Maleic acid acrylic acid copolymer	4.0
Diethylene triamine penta methylene phosphonic acid	0.4
Perborate	15.0
Tetraacetylene diamine	5.0
Smectite clay	10.0
Poly (oxy ethylene) (MW 300,000)	0.3
Ser63Glu + Thr104Asn + Gln206Ser + Tyr217Thr	0.4
Lipase	0.2
Amylase	0.3
Cellulase	0.2
Sodium silicate	3.0
Sodium carbonate	10.0
Carboxymethyl cellulose	0.2
Brighteners	0.2
Water, perfume and minors	Up to 100



Example 47  
Granular Fabric Cleaning Composition

Component	Weight %
Linear alkyl benzene sulfonate	6.92
Tallow alkyl sulfate	2.05
C <sub>14-15</sub> alcohol 7 times ethoxylated	4.4
C <sub>12-15</sub> alkyl ethoxy sulfate - 3 times ethoxylated	0.16
Zeolite	20.2
Citrate	5.5
Carbonate	15.4
Silicate	3.0
Maleic acid acrylic acid copolymer	4.0
Carboxymethyl cellulose	0.31
Soil release polymer	0.30
Asn62Ser + Trp106Gly + Ser132Asp + Ala187Ser + Phe189Ser	0.2
Lipase	0.36
Cellulase	0.13
Perborate tetrahydrate	11.64
Perborate monohydrate	8.7
Tetraacetylene diamine	5.0
Diethylene tramine penta methyl phosphonic acid	0.38
Magnesium sulfate	0.40
Brightener	0.19
Perfume, silicone, suds suppressors	0.85
Minors	Up to 100

b. Liquid fabric cleaning compositions

- 5        Liquid fabric cleaning compositions of the present invention comprise an effective amount of one or more enzyme variants of the present invention, preferably from about 0.005% to about 5%, more preferably from about 0.01% to about 1%, by weight of active enzyme of the composition. Such liquid fabric cleaning compositions typically additionally comprise an anionic surfactant, a
- 10       fatty acid, a water-soluble detergency builder and water.

The liquid fabric cleaning composition embodiment of the present invention is illustrated by the following examples.

**Examples 48-52**  
**Liquid Fabric Cleaning Compositions**

		Example No.				
Component		48	49	50	51	52
5	Ser161Glu + Gly219Asn	0.05	0.03	0.30	0.03	0.10
	Asn62Ser + Ile107Ala + Glu206Asp - + Tyr217Thr		-	-	0.01	0.20
	C <sub>12</sub> - C <sub>14</sub> alkyl sulfate, Na	20.00	20.00	20.00	20.00	20.00
	2-butyl octanoic acid	5.00	5.00	5.00	5.00	5.00
10	Sodium citrate	1.00	1.00	1.00	1.00	1.00
	C <sub>10</sub> alcohol ethoxylate (3)	13.00	13.00	13.00	13.00	13.00
	Monethanolamine	2.50	2.50	2.50	2.50	2.50
	Water/propylene glycol/ethanol (100:1:1)	balance to 100%				

15 In Examples 48-50 the BPN' variants recited in Tables 2-25, among others, are substituted for Ser161Glu + Gly219Asn, with substantially similar results.

20 In Examples 51-52, any combination of the BPN' variants recited in Tables 2-25, among others, are substituted for Ser161Glu + Gly219Asn and Asn62Ser + Ile107Ala + Glu206Asp + Tyr217Thr, with substantially similar results.

**Examples 53-57**  
**Liquid Fabric Cleaning Compositions**

		Example No.				
Component		53	54	55	56	57
25	Ser101Asp + Ile 107Ala + Gly202Ser	0.05	0.03	0.30	0.03	0.10
	Val95Thr + Thr208Gly	-	-	-	0.01	0.20
	C <sub>12</sub> - C <sub>14</sub> alkyl sulfate, Na	20.00	20.00	20.00	20.00	20.00
	2-butyl octanoic acid	5.00	5.00	5.00	5.00	5.00
30	Sodium citrate	1.00	1.00	1.00	1.00	1.00
	C <sub>10</sub> alcohol ethoxylate (3)	13.00	13.00	13.00	13.00	13.00
	Monethanolamine	2.50	2.50	2.50	2.50	2.50
	Water/propylene glycol/ethanol (100:1:1)	balance to 100%				

35 In Examples 53-55 the BPN' variants recited in Tables 2-25, among others, are substituted for Ser101Asp + Ile 107Ala + Gly202Ser, with

substantially similar results.

In Examples 56-57, any combination of the BPN' variants recited in Tables 212, among others, are substituted for Ser101Asp + Ile 107Ala + Gly202Ser and Val95Thr + Thr208Gly, with substantially similar results.

5

## Examples 58-59

## Granular Fabric Cleaning Composition

	Component	Example No.	
		58	59
	C <sub>12-14</sub> alkenyl succinic acid	3.0	8.0
10	Citric acid monohydrate	10.0	15.0
	Sodium C <sub>12-15</sub> alkyl sulphate	8.0	8.0
	Sodium sulfate of C <sub>12-15</sub> alcohol 2 times ethoxylated	-	3.0
	C <sub>12-15</sub> alcohol 7 times ethoxylated	-	8.0
	C <sub>12-15</sub> alcohol 5 times ethoxylated	8.0	-
15	Diethylene triamine penta (methylene phosphonic acid)0.2	-	-
	Oleic acid	1.8	-
	Ethanol	4.0	4.0
	Propanediol	2.0	2.0
	Asp60Glu + Gln206Asn	0.2	0.2
20	Polyvinyl pyrrolidone	1.0	2.0
	Suds suppressor	0.15	0.15
	NaOH	up to pH 7.5	
	Perborate	0.5	1
	Phenol sulphonate	0.1	0.2
25	Peroxidase	0.4	0.1
	Waters and minors	up to 100 parts	

In each of Examples 58 and 59 herein, the BPN' variants recited in Tables 2-25, among others, are substituted for Asp60Glu + Gln206Asn, with substantially similar results.

**Examples 60-62**  
**Liquid Fabric Cleaning Composition**

		Example No.		
Component		60	61	62
5	Citric Acid	7.10	3.00	3.00
	Fatty Acid	2.00	-	2.00
	Ethanol	1.93	3.20	3.20
	Boric Acid	2.22	3.50	3.50
	Monoethanolamine	0.71	1.09	1.09
10	1,2 Propanediol	7.89	8.00	8.00
	NaCumene Sulfonate	1.80	3.00	3.00
	NaFormate	0.08	0.08	0.08
	NaOH	6.70	3.80	3.80
	Silicon anti-foam agent	1.16	1.18	1.18
15	Asn61Glu	0.0145	-	-
	Gly97Glu + Thr164Pro	-	0.0145	-
	Asn62Glu + Thr158Ser + Gly215Ser	-	-	0.0145
	Lipase	0.200	0.200	0.200
	Cellulase	-	7.50	7.50
20	Soil release polymer	0.29	0.15	0.15
	Anti-foaming agents	0.06	0.085	0.085
	Brightener 36	0.095	-	-
	Brightener 3	-	0.05	0.05
	C <sub>12</sub> alkyl benzenesulfonic acid	9.86	-	-
25	C <sub>12-15</sub> alkyl polyethoxylate (2.5) sulfate	13.80	18.00	18.00
	C <sub>12</sub> glucose amide	-	5.00	5.00
	C <sub>12-13</sub> alkyl polyethoxylate (9)	2.00	2.00	2.00
	Water, perfume and minors	balance to 100%		

**c. Bar fabric cleaning compositions**

30 Bar fabric cleaning compositions of the present invention suitable for hand-washing soiled fabrics contain an effective amount of one or more enzyme variants of the present invention, preferably from about 0.001% to about 10%, more preferably from about 0.01% to about 1% by weight of the composition.

35 The bar fabric cleaning composition embodiment of the present invention is illustrated by the following examples.

**Examples 63-66**  
**Bar Fabric Cleaning Compositions**

		Example No.			
Component		63	64	65	66
5	Gly97Glu + Thr164Pro	0.3	-	0.1	0.02
	Ala98Ser + Gly154Asn	-	-	0.4	0.03
	C <sub>12</sub> -C <sub>16</sub> alkyl sulfate, Na	20.0	20.0	20.0	20.00
	C <sub>12</sub> -C <sub>14</sub> N-methyl glucamide	5.0	5.0	5.0	5.00
	C <sub>11</sub> -C <sub>13</sub> alkyl benzene sulfonate, Na	10.0	10.0	10.0	10.00
10	Sodium carbonate	25.0	25.0	25.0	25.00
	Sodium pyrophosphate	7.0	7.0	7.0	7.00
	Sodium tripolyphosphate	7.0	7.0	7.0	7.00
	Zeolite A (0.1-.10 $\mu$ )	5.0	5.0	5.0	5.00
	Carboxymethylcellulose	0.2	0.2	0.2	0.20
15	Polyacrylate (MW 1400)	0.2	0.2	0.2	0.20
	Coconut monethanolamide	5.0	5.0	5.0	5.00
	Brightener, perfume	0.2	0.2	0.2	0.20
	CaSO <sub>4</sub>	1.0	1.0	1.0	1.00
	MgSO <sub>4</sub>	1.0	1.0	1.0	1.00
20	Water	4.0	4.0	4.0	4.00
	Filler*	balance to 100%			

\*Can be selected from convenient materials such as CaCO<sub>3</sub>, talc, clay, silicates, and the like.

In Examples 63-64 the BPN' variants recited in Tables 2-25, among others, are substituted for Gly97Glu + Thr164Pro, with substantially similar results.

In Examples 65-66, any combination of the BPN' variants recited in Tables 2-25, among others, are substituted for Gly97Glu + Ghr164Pro and Ala98Ser + Gly154Asn, with substantially similar results.

**Examples 67-70**  
**Bar Fabric Cleaning Compositions**

		Example No.			
Component		67	68	69	70
5	Val203Glu	0.3	-	0.1	0.02
	Gly100Glu + Ile107Ser	-	0.3	0.4	0.03
	C <sub>12</sub> -C <sub>16</sub> alkyl sulfate, Na	20.0	20.0	20.0	20.00
	C <sub>12</sub> -C <sub>14</sub> N-methyl glucamide	5.0	5.0	5.0	5.00
	C <sub>11</sub> -C <sub>13</sub> alkyl benzene sulfonate, Na	10.0	10.0	10.0	10.00
10	Sodium carbonate	25.0	25.0	25.0	25.00
	Sodium pyrophosphate	7.0	7.0	7.0	7.00
	Sodium tripolyphosphate	7.0	7.0	7.0	7.00
	Zeolite A (0.1-10 $\mu$ )	5.0	5.0	5.0	5.00
	Carboxymethylcellulose	0.2	0.2	0.2	0.20
15	Polyacrylate (MW 1400)	0.2	0.2	0.2	0.20
	Coconut monethanolamide	5.0	5.0	5.0	5.00
	Brightener, perfume	0.2	0.2	0.2	0.20
	CaSO <sub>4</sub>	1.0	1.0	1.0	1.00
	MgSO <sub>4</sub>	1.0	1.0	1.0	1.00
20	Water	4.0	4.0	4.0	4.00
	Filler*	balance to 100%			

\*Can be selected from convenient materials such as CaCO<sub>3</sub>, talc, clay, silicates, and the like.

In Example 67, the BPN' variants recited in Tables 2-25, among others, are substituted for Val203Glu, with substantially similar results.

In Example 68, the BPN' variants recited in Tables 2-25, among others, are substituted for Gly100Glu + Ile107Ser, with substantially similar results.

In Examples 69-70, any combination of the BPN' variants recited in Tables 2-25, among others, are substituted for Val203Glu and Gly100Glu + Ile107Ser, with substantially similar results.

**B. Additional Cleaning Compositions**

In addition to the hard surface cleaning, dishwashing and fabric cleaning compositions discussed above, one or more enzyme variants of the present invention may be incorporated into a variety of other cleaning compositions where hydrolysis of an insoluble substrate is desired. Such additional cleaning

compositions include but are not limited to, oral cleaning compositions, denture cleaning compositions, and contact lens cleaning compositions.

1. Oral cleaning compositions

In another embodiment of the present invention, a pharmaceutically-  
5 acceptable amount of one or more enzyme variants of the present invention are  
included in compositions useful for removing proteinaceous stains from teeth or  
dentures. As used herein, "oral cleaning compositions" refers to dentifrices,  
toothpastes, toothgels, toothpowders, mouthwashes, mouth sprays, mouth gels,  
chewing gums, lozenges, sachets, tablets, biogels, prophylaxis pastes, dental  
10 treatment solutions, and the like. Preferably, the oral cleaning compositions  
comprise from about 0.0001% to about 20% of one or more enzyme variants of  
the present invention, more preferably from about 0.001% to about 10%, more  
preferably still from about 0.01% to about 5%, by weight of the composition, and  
a pharmaceutically-acceptable carrier. As used herein, "pharmaceutically-  
15 acceptable" means that drugs, medicaments or inert ingredients which the term  
describes are suitable for use in contact with the tissues of humans and lower  
animals without undue toxicity, incompatibility, instability, irritation, allergic  
response, and the like, commensurate with a reasonable benefit/risk ratio.

Typically, the pharmaceutically-acceptable oral cleaning carrier  
20 components of the oral cleaning compositions of the oral cleaning compositions  
will generally comprise from about 50% to about 99.99%, preferably from about  
65% to about 99.99%, more preferably from about 65% to about 99%, by weight  
of the composition.

The pharmaceutically-acceptable carrier components and optional  
25 components which may be included in the oral cleaning compositions of the  
present invention are well known to those skilled in the art. A wide variety of  
composition types, carrier components and optional components useful in the  
oral cleaning compositions are disclosed in U.S. Patent 5,096,700, Seibel,  
issued March 17, 1992; U.S. Patent 5,028,414, Sampathkumar, issued July 2,  
1991; and U.S. Patent 5,028,415, Benedict, Bush and Sunberg, issued July 2,  
30 1991; all of which are incorporated herein by reference.

The oral cleaning composition embodiment of the present invention is  
illustrated by the following examples.

**Examples 71-74**  
**Dentifrice Composition**

		Example No.			
Component		71	72	73	74
5	Gln59Asp + Ala98Glu + Gly102Asp +Ser105Glu + Leu109Thr	2.000	3.500	1.500	2.000
	Sorbitol (70% aqueous solution)	35.000	35.000	35.000	35.000
	PEG-6*	1.000	1.000	1.000	1.000
	Silica dental abrasive**	20.000	20.000	20.000	20.000
10	Sodium fluoride	0.243	0.243	0.243	0.243
	Titanium dioxide	0.500	0.500	0.500	0.500
	Sodium saccharin	0.286	0.286	0.286	0.286
	Sodium alkyl sulfate (27.9% aqueous solution)	4.000	4.000	4.000	4.000
15	Flavor	1.040	1.040	1.040	1.040
	Carboxyvinyl Polymer***	0.300	0.300	0.300	0.300
	Carrageenan****	0.800	0.800	0.800	0.800
	Water	balance to 100%			

\*PEG-6 = Polyethylene glycol having a molecular weight of 600.

20 \*\*Precipitated silica identified as Zeodent 119 offered by J.M. Huber.

\*\*\*Carbopol offered by B.F. Goodrich Chemical Company.

\*\*\*\*Iota Carrageenan offered by Hercules Chemical Company.

In Examples 71-74 the BPN' variants recited in Tables 2-25, among  
others, are substituted for Gln59Asp + Ala98Glu + Gly102Asp + Ser105Glu +  
25 Leu209Thr, with substantially similar results.



**Examples 75-78**  
**Mouthwash Composition**

		Example No.			
Component		75	76	77	78
5	Leu96Thr + Gly128Asp + Ala133Glu + Asn155Glu + Lys213Asp + Ala216Asp	3.00	7.50	1.00	5.00
	SDA 40 Alcohol	8.00	8.00	8.00	8.00
	Flavor	0.08	0.08	0.08	0.08
10	Emulsifier	0.08	0.08	0.08	0.08
	Sodium Fluoride	0.05	0.05	0.05	0.05
	Glycerin	10.00	10.00	10.00	10.00
	Sweetener	0.02	0.02	0.02	0.02
	Benzoic acid	0.05	0.05	0.05	0.05
15	Sodium hydroxide	0.20	0.20	0.20	0.20
	Dye	0.04	0.04	0.04	0.04
	Water	balance to 100%			

In Examples 75-78, the BPN' variants recited in Tables 2-25, among others, are substituted for Leu96Thr + Gly128Asp + Ala133Glu + Asn155Glu + Lys213Asp + Ala216Asp, with substantially similar results.

**Examples 79-82**  
**Lozenge Composition**

		Example No.			
Component		79	80	81	82
25	Ser132Asp + Tyr217Leu	0.01	0.03	0.10	0.02
	Sorbitol	17.50	17.50	17.50	17.50
	Mannitol	17.50	17.50	17.50	17.50
	Starch	13.60	13.60	13.60	13.60
	Sweetener	1.20	1.20	1.20	1.20
30	Flavor	11.70	11.70	11.70	11.70
	Color	0.10	0.10	0.10	0.10
	Corn Syrup	balance to 100%			

In Examples 79-82, the BPN' variants recited in Tables 2-25, among others, are substituted for Ser132Asp + Tyr217Leu, with substantially similar results.

**Examples 83-86**  
**Chewing Gum Composition**

	Component	Example No.			
		83	84	85	86
5	Thr66Pro + Gln103Asn + Lys213Asp	0.03	0.02	0.10	0.05
	Sorbitol crystals	38.44	38.40	38.40	38.40
	Paloja-T gum base*	20.00	20.00	20.00	20.00
	Sorbitol (70% aqueous solution)	22.00	22.00	22.00	22.00
	Mannitol	10.00	10.00	10.00	10.00
10	Glycerine	7.56	7.56	7.56	7.56
	Flavor	1.00	1.00	1.00	1.00

\*Supplied by L.A. Dreyfus Company.

In Examples 83-86, the BPN' variants recited in Tables 2-25, among others, are substituted for Thr66Pro + Gln103Asn + Lys213Asp, with substantially similar results.

**2. Denture cleaning compositions**

In another embodiment of the present invention, denture cleaning compositions for cleaning dentures outside of the oral cavity comprise one or more enzyme variants of the present invention. Such denture cleaning compositions comprise an effective amount of one or more of the enzyme variants, preferably from about 0.0001% to about 50% of one or more of the enzyme variants, more preferably from about 0.001% to about 35%, more preferably still from about 0.01% to about 20%, by weight of the composition, and a denture cleansing carrier. Various denture cleansing composition formats such as effervescent tablets and the like are well known in the art (see for example U.S. Patent 5,055,305, Young, incorporated herein by reference), and are generally appropriate for incorporation of one or more of the enzyme variants for removing proteinaceous stains from dentures.

The denture cleaning composition embodiment of the present invention is illustrated by the following examples.

Examples 87-90  
Two-layer Effervescent Denture Cleansing Tablet

		Example No.			
Component		87	88	89	90
5	<b><u>Acidic Layer</u></b>				
	Gln59Glu + Ser63Glu + Val95Met + Gly97Pro + Tyr217Ala	1.0	1.5	0.01	0.05
	Tartaric acid	24.0	24.0	24.00	24.00
	Sodium carbonate	4.0	4.0	4.00	4.00
10	Sulphamic acid	10.0	10.0	10.00	10.00
	PEG 20,000	4.0	4.0	4.00	4.00
	Sodium bicarbonate	24.5	24.5	24.50	24.50
	Potassium persulfate	15.0	15.0	15.00	15.00
	Sodium acid pyrophosphate	7.0	7.0	7.00	7.00
15	Pyrogenic silica	2.0	2.0	2.00	2.00
	TAED*	7.0	7.0	7.00	7.00
	Ricinoleylsulfosuccinate	0.5	0.5	0.50	0.50
	Flavor	1.0	1.0	1.00	1.00
	<b><u>Alkaline Layer</u></b>				
20	Sodium perborate monohydrate	32.0	32.0	32.00	32.00
	Sodium bicarbonate	19.0	19.0	19.00	19.00
	EDTA	3.0	3.0	3.00	3.00
	Sodium tripolyphosphate	12.0	12.0	12.00	12.00
	PEG 20,000	2.0	2.0	2.00	2.00
25	Potassium persulfate	26.0	26.0	26.00	26.00
	Sodium carbonate	2.0	2.0	2.00	2.00
	Pyrogenic silica	2.0	2.0	2.00	2.00
	Dye/flavor	2.0	2.0	2.00	2.00

\*Tetraacetylene diamine

30 In Examples 87-90, the BPN' variants recited in Tables 2-25, among others, are substituted for Gln59Glu + Ser63Glu + Val95Met + Gly97Pro + Tyr217Ala, with substantially similar results.

### 3. Contact Lens Cleaning Compositions

35 In another embodiment of the present invention, contact lens cleaning compositions comprise one or more enzyme variants of the present invention.

Such contact lens cleaning compositions comprise an effective amount of one or more of the enzyme variants, preferably from about 0.01% to about 50% of one or more of the enzyme variants, more preferably from about 0.01% to about 20%, more preferably still from about 1% to about 5%, by weight of the composition, and a contact lens cleaning carrier. Various contact lens cleaning composition formats such as tablets, liquids and the like are well known in the art (see for example U.S. Patent 4,863,627, Davies, Meaken and Rees, issued September 5, 1989; U.S. Patent Re. 32,672, Huth, Lam and Kirai, reissued May 24, 1988; U.S. Patent 4,609,493, Schäfer, issued September 2, 1986; U.S. Patent, 4,690,793, Ogunbiyi and Smith, issued September 1, 1987; U.S. Patent 4,614,549, Ogunbiyi, Riedhammer and Smith, issued September 30, 1986; and U.S. Patent 4,285,738, Ogata, issued August 25, 1981; each of which are incorporated herein by reference), and are generally appropriate for incorporation of one or more enzyme variants of the present invention for removing proteinaceous stains from contact lens.

The contact lens cleaning composition embodiment of the present invention is illustrated by the following examples.

#### Examples 91-94

##### Enzymatic Contact Lens Cleaning Solution

Component	Example No.			
	91	92	93	94
Ser191Glu + Gly219Ser	0.01	0.5	0.1	2.0
Glucose	50.00	50.0	50.0	50.0
Nonionic surfactant (polyoxyethylene-polyoxypropylene copolymer)	2.00	2.0	2.0	2.0
Anionic surfactant (polyoxyethylene-alkylphenylether sodium sulfricester)	1.00	1.0	1.0	1.0
Sodium chloride	1.00	1.0	1.0	1.0
Borax	0.30	0.3	0.3	0.3
Water	balance to 100%			

In Examples 91-94, the BPN' variants recited in Tables 2-25, among others, are substituted for Ser191Glu + Gly219Ser, with substantially similar results.

While particular embodiments of the subject invention have been described, it will be obvious to those skilled in the art that various changes and

**modifications of the subject invention can be made without departing from the spirit and scope of the invention. It is intended to cover, in the appended claims, all such modifications that are within the scope of the invention.**

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- 5 (i) APPLICANT: BRODE, PHILIP F. et al.
- 10 (ii) TITLE OF INVENTION: BPN' VARIANTS HAVING DECREASED  
ADSORPTION AND INCREASED HYDROLYSIS WHEREIN ONE  
OR MORE LOOP REGIONS ARE SUBSTITUTED
- (iii) NUMBER OF SEQUENCES: 1
- 15 (iv) CORRESPONDENCE ADDRESS:  
(A) ADDRESSEE: THE PROCTER & GAMBLE COMPANY  
(B) STREET: 11810 EAST MIAMI RIVER ROAD  
(C) CITY: ROSS  
(D) STATE: OH  
(E) COUNTRY: USA  
20 (F) ZIP: 45061
- (v) COMPUTER READABLE FORM:  
(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
25 (C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:  
(A) APPLICATION NUMBER:  
30 (B) FILING DATE:  
(C) CLASSIFICATION:
- (vii) ATTORNEY/AGENT INFORMATION:  
(A) NAME: CORSTANJE, BRAHM J.  
35 (B) REGISTRATION NUMBER: 34,804  
(C) ATTORNEY DOCKET NO. 5597
- (ix) TELECOMMUNICATION INFORMATION:  
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40 (B) TELEFAX: 513-627-0260
- (2) INFORMATION FOR SEQ ID NO:1:
- 45 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 275 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear
- 50 (ii) MOLECULE TYPE: protein
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
- 55 Ala Gln Ser Val Pro Tyr Gly Val Ser Gln Ile Lys Ala Pro Ala Leu  
1 5 10 15
- His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val Ile Asp  
60 20 25 30
- Ser Gly Ile Asp Ser Ser His Pro Asp Leu Lys Val Ala Gly Gly Ala  
35 40 45

Ser Met Val Pro Ser Glu Thr Asn Pro Phe Gln Asp Asn Asn Ser His  
 50 55 60  
 5 Gly Thr His Val Ala Gly Thr Val Ala Ala Leu Asn Asn Ser Ile Gly  
 65 70 75 80  
 Val Leu Gly Val Ala Pro Ser Ala Ser Leu Tyr Ala Val Lys Val Leu  
 85 90 95  
 10 Gly Ala Asp Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly Ile Glu  
 100 105 110  
 Trp Ala Ile Ala Asn Asn Met Asp Val Ile Asn Met Ser Leu Gly Gly  
 115 120 125  
 15 Pro Ser Gly Ser Ala Ala Leu Lys Ala Ala Val Asp Lys Ala Val Ala  
 130 135 140  
 Ser Gly Val Val Val Val Ala Ala Ala Gly Asn Glu Gly Thr Ser Gly  
 145 150 155 160  
 Ser Ser Ser Thr Val Gly Tyr Pro Gly Lys Tyr Pro Ser Val Ile Ala  
 165 170 175  
 25 Val Gly Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Val  
 180 185 190  
 Gly Pro Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr  
 195 200 205  
 30 Leu Pro Gly Asn Lys Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala Ser  
 210 215 220  
 Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn  
 225 230 235 240  
 Trp Thr Asn Thr Gln Val Arg Ser Ser Leu Glu Asn Thr Thr Thr Lys  
 245 250 255  
 40 Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val Gln Ala  
 260 265 270  
 Ala Ala Gln  
 275  
 45

What is claimed is:

1. A BPN' variant having a modified amino acid sequence of wild-type amino acid sequence, the wild-type amino acid sequence comprising a first loop region, a second loop region, a third loop region, a fourth loop region and a fifth loop region; characterized in that the modified amino acid sequence comprises a substitution at one or more positions in one or more of the loop regions; wherein

- A. when a substitution occurs in the first loop region, the substitution occurs at one or more of positions 59, 60, 61, 62, 63, 65 or 66; wherein
  - a. when a substitution occurs at position 59, the substituting amino acid is Asn, Asp, Glu or Ser;
  - b. when a substitution occurs at position 60, the substituting amino acid is Glu;
  - c. when a substitution occurs at position 61, the substituting amino acid is Asp, Gln, Glu or Ser;
  - d. when a substitution occurs at position 62, the substituting amino acid is Asp, Gln, Glu or Ser;
  - e. when a substitution occurs at position 63, the substituting amino acid is Asp or Glu;
  - f. when a substitution occurs at position 65, the substituting amino acid is Asn, Asp, Gln, Glu, Pro or Ser; and
  - g. when a substitution occurs at position 66, the substituting amino acid is Asn, Asp, Gln, Glu, Gly, Pro or Ser;
- B. when a substitution occurs in the second loop region, the substitution occurs at one or more of positions 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106 or 107; wherein
  - a. when a substitution occurs at position 95, the substituting amino acid is Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Met, Pro, Ser or Thr;
  - b. when a substitution occurs at position 96, the substituting amino acid is Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Met, Pro, Ser, Thr or Val;
  - c. when a substitution occurs at position 97, the substituting amino acid is Asn, Asp, Gln, Glu, Pro or Ser;



- d. when a substitution occurs at position 98, the substituting amino acid is Asn, Asp, Gln, Glu, Gly, His, Pro, Ser or Thr;
  - e. when a substitution occurs at position 99, the substituting amino acid is Glu;
  - f. when a substitution occurs at position 100, the substituting amino acid is Asn, Asp, Gln, Glu, Pro or Ser;
  - g. when a substitution occurs at position 101, the substituting amino acid is Asp or Glu;
  - h. when a substitution occurs at position 102, the substituting amino acid is Asn, Asp, Gln, Glu, Pro or Ser;
  - i. when a substitution occurs at position 103, the substituting amino acid is Asn, Asp, Glu or Ser;
  - j. when a substitution occurs at position 104, the substituting amino acid is Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Met, Pro, Ser, Thr or Val;
  - k. when a substitution occurs at position 105, the substituting amino acid is Asp or Glu;
  - l. when a substitution occurs at position 106, the substituting amino acid is Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Pro, Ser, Thr, Tyr or Val; and
  - m. when a substitution occurs at position 107, the substituting amino acid is Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Leu, Met, Pro, Ser, Thr or Val;
- C. when a substitution occurs in the third loop region, the substitution occurs at one or more of positions 126, 127, 128, 129, 130, 131, 132 or 133; wherein
- a. when a substitution occurs at position 126, the substituting amino acid is Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Met, Pro, Ser, Thr or Val;
  - b. when a substitution occurs at position 127, the substituting amino acid is Asn, Asp, Gln, Glu, Pro or Ser;
  - c. when a substitution occurs at position 128, the substituting amino acid is Asn, Asp, Gln, Glu, Gly or Ser;
  - d. when a substitution occurs at position 129, the substituting amino acid is Asn, Asp, Gln, Glu, Gly or Ser;
  - e. when a substitution occurs at position 130, the substituting amino acid is Asp or Glu;

- f. when a substitution occurs at position 131, the substituting amino acid is Asn, Asp, Gln, Glu, Gly or Ser;
- g. when a substitution occurs at position 132, the substituting amino acid is Asp or Glu; and
- h. when a substitution occurs at position 133, the substituting amino acid is Asn, Asp, Gln, Glu, Gly, His, Pro, Ser or Thr;
- D. when a substitution occurs in the fourth loop region, the substitution occurs at one or more of positions 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166 or 167; wherein
  - a. when a substitution occurs at position 154, the substituting amino acid is Asn, Asp, Gln, Glu, Pro or Ser;
  - b. when a substitution occurs at position 155, the substituting amino acid is Asp, Gln, Glu or Ser;
  - c. when a substitution occurs at position 156, the substituting amino acid is Asp;
  - d. when a substitution occurs at position 157, the substituting amino acid is Asn, Asp, Gln, Glu, Pro or Ser;
  - e. when a substitution occurs at position 158, the substituting amino acid is Asn, Asp, Gln, Glu, Gly, Pro or Ser;
  - f. when a substitution occurs at position 159, the substituting amino acid is Asp or Glu;
  - g. when a substitution occurs at position 160, the substituting amino acid is Asn, Asp, Gln, Glu, Pro or Ser;
  - h. when a substitution occurs at position 161, the substituting amino acid is Asp or Glu;
  - i. when a substitution occurs at position 162, the substituting amino acid is Asp or Glu;
  - j. when a substitution occurs at position 163, the substituting amino acid is Asp or Glu;
  - k. when a substitution occurs at position 164, the substituting amino acid is Asn, Asp, Gln, Glu, Gly, Pro or Ser;
  - l. when a substitution occurs at position 165, the substituting amino acid is Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Met, Pro, Ser or Thr;
  - m. when a substitution occurs at position 166, the substituting amino acid is Asn, Asp, Gln, Glu, Pro or Ser; and

- n. when a substitution occurs at position 167, the substituting amino acid is Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Met, Pro, Ser, Thr or Val; and
- E. when a substitution occurs in the fifth loop region, the substitution occurs at one or more of positions 187, 188, 189, 190 or 191; wherein
  - a. when a substitution occurs at position 187, the substituting amino acid is Asn, Asp, Gln, Glu, Gly, His, Pro, Ser and Thr;
  - b. when a substitution occurs at position 188, the substituting amino acid is Asp or Glu;
  - c. when a substitution occurs at position 189, the substituting amino acid is Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Met, Pro, Ser, Thr, Tyr or Val;
  - d. when a substitution occurs at position 190, the substituting amino acid is Asp or Glu; and
  - e. when a substitution occurs at position 191, the substituting amino acid is Asp or Glu;

whereby the BPN' variant has decreased adsorption to, and increased hydrolysis of, an insoluble substrate as compared to wild-type subtilisin BPN'.

- 2. The BPN' variant of Claim 1, wherein one or more substitutions occur in the first loop region.
- 3. The BPN' variant of Claim 1, wherein one or more substitutions occur in the second loop region.
- 4. The BPN' variant of Claim 1, wherein one or more substitutions occur in the third loop region.
- 5. The BPN' variant of Claim 1, wherein one or more substitutions occur in the fourth loop region.
- 6. The BPN' variant of Claim 1, wherein one or more substitutions occur in the fifth loop region.
- 7. The BPN' variant of any of Claims 1-6, wherein the wild-type amino acid sequence further comprises a sixth loop region, characterized in that the

modified amino acid sequence further comprises one or more substitutions in the sixth loop region; wherein the substitution(s) in the sixth loop region occurs at one or more of positions 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219 or 220; wherein

- a. when a substitution occurs at position 199, the substituting amino acid for position 199 is Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu;
- b. when a substitution occurs at position 200, the substituting amino acid for position 200 is His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu;
- c. when a substitution occurs at position 201, the substituting amino acid for position 201 is Gly, Gln, Asn, Ser, Asp or Glu;
- d. when a substitution occurs at position 202, the substituting amino acid for position 202 is Pro, Gln, Asn, Ser, Asp or Glu;
- e. when a substitution occurs at position 203, the substituting amino acid for position 203 is Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu;
- f. when a substitution occurs at position 204, the substituting amino acid for position 204 is Asp, or Glu;
- g. when a substitution occurs at position 205, the substituting amino acid for position 205 is Leu, Val, Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu;
- h. when a substitution occurs at position 206, the substituting amino acid for position 206 is Pro, Asn, Ser, Asp, or Glu;
- i. when a substitution occurs at position 207, the substituting amino acid for position 207 is Asp or Glu;
- j. when a substitution occurs at position 208, the substituting amino acid for position 208 is Pro, Gly, Gln, Asn, Ser, Asp or Glu;
- k. when a substitution occurs at position 209, the substituting amino acid for position 209 is Ile, Val, Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu;
- l. when a substitution occurs at position 210, the substituting amino acid for position 210 is Ala, Gly, Gln, Asn, Ser, Asp or Glu;
- m. when a substitution occurs at position 211, the substituting amino acid for position 211 is Ala, Pro, Gln, Asn, Ser, Asp or Glu;
- n. when a substitution occurs at position 212, the substituting amino acid for position 212 is Gln, Ser, Asp or Glu;

- o. when a substitution occurs at position 213, the substituting amino acid for position 213 is Trp, Phe, Tyr, Leu, Ile, Val, Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu;
  - p. when a substitution occurs at position 214, the substituting amino acid for position 214 is Phe, Leu, Ile, Val, Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu;
  - q. when a substitution occurs at position 215, the substituting amino acid for position 215 is Thr, Pro, Gln, Asn, Ser, Asp or Glu;
  - r. when a substitution occurs at position 216, the substituting amino acid for position 216 is His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu;
  - s. when a substitution occurs at position 217, the substituting amino acid for position 217 is Leu, Ile, Val, Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu;
  - t. when a substitution occurs at position 218, the substituting amino acid for position 218 is Gln, Ser, Asp or Glu;
  - u. when a substitution occurs at position 219, the substituting amino acid for position 219 is Pro, Gln, Asn, Ser, Asp or Glu; and
  - v. when a substitution occurs at position 220, the substituting amino acid for position 220 is Pro, Gly, Gln, Asn, Ser Asp or Glu.
8. A cleaning composition selected from the group consisting of a hard surface cleaning composition, a dishwashing composition, an oral cleaning composition, a denture cleansing composition, a contact lens cleaning composition and a fabric cleaning composition, characterized in that the cleaning composition comprises the BPN' variant of any of Claims 1-7 and a cleaning composition carrier.
9. The cleaning composition of Claim 8, wherein the cleaning composition is a hard surface cleaning composition.
10. The cleaning composition of Claim 8, wherein the cleaning composition is a fabric cleaning composition.
11. A mutant BPN' gene encoding the BPN' variant of any of Claims 1-7.

# INTERNATIONAL SEARCH REPORT

Inter. Appl. No.

PCT/US 95/03176

**A. CLASSIFICATION OF SUBJECT MATTER**  
 IPC 6 C12N15/57 C11D3/386 C12N9/54

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
 IPC 6 C12N C11D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP-A-0 405 901 (UNILEVER PLC ;UNILEVER NV (NL)) 2 January 1991 see claims	1-5,7-11
X	WO-A-94 02618 (GIST BROCADES NV ;MULLENERS LEONARDUS JOHANNES S (NL); MISSET ONNO) 3 February 1994 see tables II , III	1,3-5, 7-11
X	WO-A-89 09830 (GENEX CORP) 19 October 1989 see claims; table 2	1,3,7-11
X	WO-A-87 05050 (GENEX CORP) 27 August 1987 see page 18; claims	1,4,5, 7-11
	-/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"A" document member of the same patent family

Date of the actual completion of the international search

27 July 1995

Date of mailing of the international search report

16.08.95

Name and mailing address of the ISA

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Authorized officer

Van der Schaal, C

# INTERNATIONAL SEARCH REPORT

Int. Application No  
PCT/US 95/03176

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP-A-0 328 229 (GIST BROCADES NV) 16 August 1989 see example 12 -----	1,5,7-11
A	CHEMICAL ABSTRACTS, vol. 116, no. 23, 8 June 1992 Columbus, Ohio, US; abstract no. 230623, P. BRODE AND D. RAUCH 'Subtilisin BPN' activity on an immobilized substrate' cited in the application see abstract & LANGMUIR, vol. 8, no. 5, 1992 pages 1325-1329, -----	

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International Application No

**PCT/US 95/03176**

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0405901	02-01-91	WO-A-	9100334	10-01-91
		JP-T-	4500385	23-01-92
		EP-A-	0405902	02-01-91
		WO-A-	9100335	10-01-91
		JP-T-	4500384	23-01-92
WO-A-9402618	03-02-94	AU-B-	4700693	14-02-94
		CA-A-	2139928	03-02-94
		FI-A-	950168	10-03-95
WO-A-8909830	19-10-89	US-A-	5013657	07-05-91
		EP-A-	0409878	30-01-91
		JP-T-	3503602	15-08-91
		US-A-	4990452	05-02-91
		US-A-	5246849	21-09-93
WO-A-8705050	27-08-87	EP-A-	0260299	23-03-88
		JP-T-	63502959	02-11-88
		US-A-	4980288	25-12-90
		US-A-	4990452	05-02-91
EP-A-0328229	16-08-89	AU-B-	629814	15-10-92
		AU-A-	3050189	06-09-89
		DE-D-	68912359	03-03-94
		DE-T-	68912359	09-06-94
		EP-A-	0571049	24-11-93
		ES-T-	2061929	16-12-94
		JP-T-	2503986	22-11-90
		WO-A-	8907642	24-08-89
		PT-B-	89702	29-04-94
		US-A-	5336611	09-08-94
		US-A-	5324653	28-06-94